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THE MICROTOMIST'S VADE-MECUM

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MICROTOMIST'S VADE-MECUM

A HANDBOOK OF THE METHODS OF MICROSCOPIC ANATOMY

BY

ARTHUR BOLLES LEE

THIRD EDITION

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PREFACE

THE present edition of this work has been most thoroughly revised, and has been brought up to date by the incorporation of all the new matter of any value that has appeared since the publication of the second edition.

Notwithstanding the very great pains that have been taken in selecting matter for omission,* and in condensing the text as far as practicable, the book turns out to be considerably enlarged. The reason for this is that the last three years have produced, together with the usual crop of ignorant futilities, a large amount of really valuable new matter, of which due account has been rendered.

Amongst this new matter I would direct the reader's attention to the thoroughly scientific and successful work on the Theory of Staining with Carmine and with Hæmatoxylin, done by Paul Mayer, summarised in Chapters X and XI, and to the new formulæ for stains resulting therefrom. The results are eminently satisfactory, both theoretically and practically, and constitute the most brilliant and solid contribution to this branch of the subject that has been made since the publication, fourteen years ago, of Grenacher's epoch-making alum-carmine and borax-carmine, a feat that has not since been equalled.

The Ehrlich-Biondi-Heidenhain staining mixture may also be noticed as being in its way a most brilliantly successful thing; it has in the course of the last three years attained an almost unexampled degree of popularity.

As in the last edition, great extension has been given to the subject of Neurological Methods, which now cover over sixty pages, exclusive of the Methylen Blue method and other impregnation methods discussed in Part I. This

* The receipt for Beale's carmine has been rejected from the text. Probably the older among my readers will note its disappearance with some touch of emotion. *Eheu, fugaces!*

great extension is in large measure due to the luxuriant growth of the literature that has sprung up round the hæmatoxylin methods of Weigert and the bichromate-of-silver method of Golgi, and which it has been deemed advisable to treat with all possible fulness.

The Methylen Blue impregnation method has nearly an entire chapter assigned to it. This important process has by no means been brought to perfection, but much has been done already, and the future is full of promise.

The progress that has been made during the last three years is certainly gratifying, but I must once more complain of the mischief that is done by the method-mongers. The reckless publication of crude or needless histological methods is a crying evil. The really useful matter that is published is smothered in a sea of rubbish, and the investigator is obliged to devote to the acquisition of such new technical knowledge as he may require very much more time than would suffice if he were only unimpeded. Some idea of the magnitude of the evil may perhaps be gleaned from the fact that this book has now been out of print for many months, the mass of literature that it has been necessary to digest for the purpose of a new edition being so great that the appearance of the work has been delayed far beyond the contemplated time.

It is useless to remonstrate with the persons of whom I am complaining; they cannot grasp the fact of their ignorance, and cannot be brought to see that nobody heeds them. I would appeal earnestly, therefore, to those with whom the power lies to put an end to this nuisance. It surely is not too much to ask that the editors of the periodicals devoted to these matters should refuse to publish processes that are not demonstrably new, and that do not make out a good claim to be useful.

Nyon, Switzerland;
August, 1893.

PREFACE

TO THE

FIRST EDITION

In its primary intention this work appeals rather to the instructed anatomist than to the beginner. Its aim is to put into his hands a concise but complete account of all the methods of preparation that have been recommended as useful for the purposes of Microscopic Anatomy, and so furnish him with a ready source of information on points of detail as to which his memory or his knowledge may be at fault. This object is attained by the mere collection of Formulæ set out in Part I, and of special methods described in Part II. But the book could obviously be made to subserve a further end —that of a guide to the beginner. To this end I have added a General Introduction and a series of introductory paragraphs prefixed, where needful, to the different chapters. These introductory portions, taken together, go far to make up a formal treatise on the art. And as a further aid to the beginner I have added the collection of examples given in Part II. These examples are of course not intended for servile imitation, but rather as hints suggestive of the most fitting processes.

The collection of Formulæ here brought together is, I believe, practically exhaustive; no process having any claim to scientific status having been rejected, nor any, I trust, unwittingly omitted. It may be useful here to say a word as to the reasons for this—perhaps apparently excessive—catholicity of treatment. Doubtless a large proportion of the formulæ given are quite superseded in modern practice; but that is not a sufficient reason for rejecting them. The inclusion of all of them is justified by the consideration that some

one or other of them may perhaps serve, in some way that cannot now be foreseen, to suggest some new method of value. Let me give an example. Who, ten years ago, would have thought that the formula of Blanchard's "Liqueur saline hydrargyrique" deserved reprinting in a treatise on histologic technic? Yet it is to the disinterment of that forgotten formula by Lang that we owe the establishment of corrosive sublimate as one of the most useful fixing agents in the arsenal of the microtomist. Or who would have deemed Thiersch's lilac borax-carmine (Formula No. 80 *a*), published in 1865, to be of greater importance than any other stain till then made known? Yet that formula it was that directly suggested Woodward's admirable aqueous borax-carmine, and through this, if I am not mistaken, the aqueous and the alcoholic borax-carmines of Grenacher, the latter of which is now to be found on the table of every embryologist.

All my abstracts and translations have been made from the original sources, except where it has been impossible for me to obtain sight of these. References to the sources are given in all cases; but I desire here to make special acknowledgment of the great assistance rendered me by the *Journal of the Royal Microscopical Society*—in many respects the best edited periodical known to me.

GENEVA, SWITZERLAND;
February, 1885.

CONTENTS

PART I

PARAGRAPHS

CHAPTER I.

INTRODUCTORY.—The General Method . . . 1—5

CHAPTER II.

KILLING . . . . . 6—20
Sudden Killing, 7, 8; Narcotisation, 9—20.

CHAPTER III.

FIXING . . . . . 21—24

CHAPTER IV.

FIXING AGENTS: MINERAL ACIDS AND THEIR SALTS . 25—46

CHAPTER V.

FIXING AGENTS: CHLORIDES, ORGANIC ACIDS, AND OTHERS . . . . . 47—66

CHAPTER VI.

HARDENING AGENTS . . . . . 67—90
Introduction, 67, 68; Mineral Acids, 64—74; Salts, 75—82; Chlorides and others, 83—90.

CHAPTER VII.

STAINING . . . . . 91—94

CHAPTER VIII.

ANILIN COLOURS GIVING INDIRECT NUCLEAR STAINS (FLEMMING'S METHOD) AND DIRECT NUCLEAR STAINS . 95—112
General Directions, 96—100; Indirect Stains, 101—107; Direct Stains, 108—112.

PARAGRAPHS

CHAPTER IX.

METHYLEN BLUE AND OTHER ANILIN STAINS . . 113—143
Methylen Blue, 113—122; Plasma and Ground Stains, 123—143.

CHAPTER X.

CARMINE AND COCHINEAL STAINS . . . . . 144—173
Theory of Carmine Staining, 144—150; Aqueous Carmines, Acid, 151—158; Neutral and Alkaline, 159—167; Alcoholic Carmines and Cochineals, 168—173.

CHAPTER XI.

HÆMATEÏN (HÆMATOXYLIN) AND OTHER ORGANIC STAINS . 174—209
Theory of Staining with Hæmatoxylin, 174—179; Hæmateïn (Hæmatoxylin), 180—199; other Organic Stains, 200—209.

CHAPTER XII.

METALLIC STAINS (IMPREGNATION METHODS) . . 210—234
Silver, 212—219; Gold, 220—230; other Metallic Stains, 231—234.

CHAPTER XIII.

COMBINATION STAINS . . . . . 235—270
Combinations having Carmine for a Primary Stain, 237—249; Combinations having Hæmatoxylin for a Primary Stain, 250—257; other Combinations, 258—270.

CHAPTER XIV.

IMBEDDING METHODS: INTRODUCTION . . . 271—274

CHAPTER XV.

IMBEDDING METHODS: PARAFFIN AND OTHER FUSION MASSES . . . . . 275—296
Paraffin, 275—287; Soap, 288—291; Gelatin, 292—296.

CHAPTER XVI.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS . 297—322
Collodion or Celloidin, 297—306; other Evaporation Masses and Freezing Masses, 307—322.

PARAGRAPHS

CHAPTER XVII.

SERIAL SECTION MOUNTING . . . . . 323—341
Methods for Paraffin Sections, 324—333; Methods for Watery Sections, 334—336; Methods for Celloidin Sections, 337—341.

CHAPTER XVIII.

CLEARING AGENTS . . . . . 342—355

CHAPTER XIX.

INDIFFERENT LIQUIDS: EXAMINATION AND PRESERVATION MEDIA . . . . . 356—429
Aqueous Liquids, 356—378; Mercurial Liquids, 379—385; other Fluids, 386—402; Glycerin Media, 403—413; Resinous Media, 417—429.

CHAPTER XX.

CEMENTS AND VARNISHES . . . . . 430—456

CHAPTER XXI.

INJECTIONS: GELATIN MASSES . . . . . 457—497
Carmine, 466—473; Blue, 474—481; other Colours, 482—497.

CHAPTER XXII.

INJECTIONS: OTHER MASSES . . . . . 498—521
White of Egg, Gum, 498, 499; Glycerin, 500—506; Aqueous, 507—512; Celloidin, 513, 514; other Masses, 515—521.

CHAPTER XXIII.

MACERATION AND DIGESTION . . . . . 522—554
Maceration, 522—548; Digestion, 549—554.

CHAPTER XXIV.

CORROSION, DECALCIFICATION, AND BLEACHING . . 555—583
Corrosion, 555—558; Decalcification and Desilicification, 559—574; Bleaching, 575—583.

PART II

SPECIAL METHODS AND EXAMPLES.

PARAGRAPHS

CHAPTER XXV.

EMBRYOLOGICAL METHODS . . . . . 584—631

CHAPTER XXVI.

CYTOLOGICAL METHODS . . . . . 632—645

CHAPTER XXVII.

TEGUMENTARY ORGANS . . . . . 646—660

CHAPTER XXVIII.

MUSCLE AND TENDON (NERVE-ENDINGS) . . . 661—681
Striated Muscle, 661—670; Tendon, 671—675; Smooth Muscle, 676—681.

CHAPTER XXIX.

RETINA, INNER EAR, NERVES . . . . . 682—727
Retina, 682—685; Inner Ear, 686, 687; Nerves, 688—727.

CHAPTER XXX.

CENTRAL NERVOUS SYSTEM . . . . . 728—785
Hardening, 729—742; Imbedding and Cutting, 743; Staining, 744—776; Mounting, 777, 778; other Methods, 779—785.

CHAPTER XXXI.

SOME OTHER HISTOLOGICAL METHODS . . . . 786—811
Connective Tissues, 786—801; Blood, 802—806; Glands, 807—811.

CHAPTER XXXII.

SOME ZOOLOGICAL METHODS . . . . . 811—861
Tunicata, 811; Mollusca, 813—821; Arthropoda, 822—831; Vermes, 832—844; Echinodermata, 845—848; Cœlenterata, 849—855; Porifera, 856; Protozoa, 857—861.

APPENDIX . . . . . 862—881

INDEX . . . . . pp. 477—509

THE MICROTOMIST'S VADE-MECUM.

CHAPTER I.

INTRODUCTORY.

1. The methods of modern microscopic anatomy may be roughly classed as General and Special. There is a General or Normal method, known as the method of sections, which consists in carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in balsam. It is by this method that the work is blocked-out and very often finished. Special points are then studied, if necessary, by Special Methods, such as examination of the living tissue elements, *in situ*, or in "indifferent" media; fixation with special fixing agents; staining with special stains; dissociation by teasing or maceration; injection; impregnation; and the like.

2. The General Method.—The first thing to be done with any structure is to *fix* its histological elements. (This statement applies equally to all classes of objects, whether it be desired to cut them into sections or to treat them in any other special way.) Two things are implied by the word "fixing;" first, the rapid *killing* of the element, so that it may not have time to change the form it had during life, but is fixed in death in the attitude it normally had during life; and second, the *hardening* of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. Too much stress can hardly be laid on this point, which is the most distinctive

feature of modern histological practice; without good fixation it is impossible to get good stains, or good sections, or preparations good in any way.

The structure having been duly fixed by one of the processes described in the chapter on FIXING AGENTS, is *washed* in order to remove from the tissues as far as possible all traces of the fixing reagent.

The kind of liquid with which washing out is done is not a matter of indifference. If corrosive sublimate (for instance) or osmic acid, or a solution into which chromic acid or a chromate enters, have been used for fixing, the washing may be done with water. But if picric acid in any form has been used, the washing must be done with alcohol. The reason of this difference is that the first-named reagents (and, indeed, all the compounds of the heavy metals used for fixing) appear to enter into a state of chemical combination with the elements of tissues, rendering them insoluble in water; so that the hardening induced by these agents is not removed by subsequent treatment with water. Picric acid, on the other hand, produces only a very slight hardening of the tissues, and does not appear to enter into any combination whatever with their elements, as it is entirely removable by treating the tissues with water or alcohol. If the removal be effected by means of water, the tissue elements are left in a soft state in which they are obnoxious to all the hurtful effects of water. Alcohol must therefore be taken to remove the picric acid and to effect the necessary hardening at the same time. Instructions for washing out are given, when necessary, in the discussion of the different fixing agents in the following parts of this work.

At the same time that the superfluous fixing agent is being removed from the tissues, or as soon as that is done, the *water of the tissues must be removed.* This is necessary for two reasons; firstly, in the interest of preservation, the presence of water being the condition of all others that most favours post-mortem decomposition; and secondly, because all water must be removed in order to allow the tissues to be impregnated with the imbedding material necessary for section-cutting, or with the balsam with which they are to be finally preserved. (The cases in which aqueous imbedding and preserving media are employed are exceptional, and will be treated of in the proper places.) The *dehydration* is performed as follows: the objects are brought into weak alcohol, and are then passed through successive alcohols of gradually increased strength, remaining in each the time necessary for complete saturation. and the last bath consisting of absolute or at least very strong alcohol.

In dealing with extremely delicate objects, it may be necessary to take

special precautions in order to avoid injury to them through the violent diffusion-currents that are set up in the passage from water to alcohol, or from one bath of alcohol to another of considerably different density. Some kind of diffusion-apparatus should be used in these cases. The objects may be placed in a tube plugged at one end and closed at the other by a diaphragm of chamois skin or other suitable membrane, the tube being then immersed in a vessel containing the grade of alcohol that it is desired to add to the liquid in the tube, and the whole allowed to remain until by diffusion through the diaphragm the two liquids have become of equal density. Or COBB's differentiator (*Proc. Linn. Soc.*, N.S.W., v, 1890, p. 157; *Journ. Roy. Mic. Soc.*, 1890, p. 821) may be employed. Or, more conveniently in most cases, the apparatus described and figured by HASWELL (*Proc. Linn. Soc.*, N.S.W., vi, 1891, p. 433; *Journ. Roy. Mic. Soc.*, 1892, p. 696). This consists of two wash-bottles connected in the usual way by tubing, and furnished, the one with an overflow-tube, and the other with a feeding-tube leading from a reservoir connected with it by means of a regulating tap or drop arrangement. The objects are placed in the first bottle; some of the same liquid as that containing the objects is placed in the second bottle; and alcohol of the grade that it is desired to add is led into it from the reservoir. The mixture of liquids therefore takes place in the bottle that does not contain the objects, and the mixture itself is gradually led over to the objects through the siphon-tube connecting the two bottles. Another apparatus for rapid dehydration, devised by CHEATLE, will be found described in *Journ. Pathol. and Bacteriol.*, i, 1892, p. 253, or *Journ. Roy. Mic. Soc.*, 1892, p. 892.

It is sometimes stated that it is necessary that the last alcohol bath should consist of absolute alcohol. This however is incorrect, a strength of 90 per cent., or at all events 95 per cent., being sufficient in almost all cases. For the small amount of water that remains in the tissues after treatment with these grades of alcohol is efficiently removed in the bath of clearing agent, if a good clearing agent be employed. Oil of cedar will remove the remaining water from tissues saturated with 95 per cent. alcohol; oil of bergamot will "clear" from 90 per cent. alcohol, and anilin oil will clear from 70 per cent. alcohol.

It is not generally necessary that pure ethylic alcohol be employed for dehydration; methylated spirit will suffice in most cases.

I am not aware of any substance that can entirely take the place of alcohol for dehydration and preservation. Acetone, and methylal, have been lately (PARKER, *Zool. Anz.*, 403, 1892, p. 376) substituted for alcohol in the dehydration of methylen-blue preparations; but the great boon of an efficient substitute for alcohol in general work remains yet to be discovered.

Considered as a mere dehydrating agent, alcohol fulfils its functions fairly well. But considered as a histological preservative agent, it is far less satisfactory. If tissues be left in alcohol for only a few days before subjecting them to the further stages of preparation, the injurious effects of a sojourn in alcohol will perhaps not be very disagreeably evident. But it is otherwise if, as must often be the case, they are put away for many weeks or months before the final preparation can be carried out. The dehydrating

action of the alcohol being continuously prolonged, the minute structure of tissues is sometimes considerably altered by it; they become overhard and shrink, and become brittle, and their capacity for taking stains well becomes seriously diminished. KULTSCHITZKY (*Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 349) has proposed to remedy this by putting up objects, after fixation and washing out with alcohol, in ether, xylol, or toluol. FLEMMING has lately (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 685) advised putting up objects after fixation in a mixture of alcohol, glycerin, and water, in about equal parts, pointing out that objects thus preserved may be at any moment either prepared for sectioning by treatment with pure alcohol, or softened for dissection or teasing by a little soaking in water, and that they do not become so hard and brittle as alcohol specimens, and retain their staining power much better. After extensive experience of this plan, I can highly recommend it, and would only further suggest that the action of the liquid seems to me to be in many cases much improved by addition of a little acetic acid (say 0·5 to 0·75 per cent.).

The water having been thus sufficiently removed, the alcohol is in its turn removed from the tissues, and its place taken by some anhydrous substance, generally an essential oil, which is miscible with the material used for imbedding. This operation is known as *Clearing*. It is very important that the passage from the last alcohol to the clearing agent be made gradual. This is effected by placing the clearing medium *under* the alcohol. A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clearing medium is introduced *at the bottom of the alcohol*. Or you may first put the clearing medium into the tube, and then carefully pour the alcohol on to the top of it. The two fluids mingle but slowly. The objects to be cleared being now quietly put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and the objects will be found to be completely penetrated by the clearing medium. (It may be noted here that this method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid—for instance, from alcohol, or from water to glycerine. It is a *more exact* method than that of successive baths of mixture of alcohol and clearing agent.)

The objects are now *imbedded*. They are removed from

the clearing medium, and soaked until thoroughly saturated in the imbedding medium. This is, for small objects, generally paraffin, liquefied by heat, and for large objects generally a solution of collodion or "celloïdin." The imbedding medium containing the object is then made to solidify, as described in the chapter on imbedding processes, and sections are made with a microtome through the imbedding mass and the included objects. The sections are then mounted on a slide by one of the methods described in the chapter on Serial Section Methods, the imbedding material is removed from them (in the case of paraffin), they are stained *in situ* on the slide, dehydrated with alcohol, cleared and mounted in balsam or damar. Or they may be stained, washed, dehydrated, and cleared in watch-glasses, and afterwards mounted as desired —the imbedding medium being first removed if desirable.

It is not always desirable to remove the imbedding mass; celloidin sections stain well without being freed from it, and are usually even dehydrated, cleared, and mounted without removal of the mass, which becomes quite transparent in balsam. This plan has the advantage, which is a very important one for large sections, of allowing the sections to remain during the whole of the manipulations protected by a supporting mass that holds all their parts together.

The plan of staining sections on the slide is of somewhat recent introduction; before it had been worked out the practice was to stain structures *in toto*, before cutting sections. And in cases in which structures are sufficiently small and permeable to allow of satisfactory staining in this way, and if it be not essential to save time, this plan is sometimes as good as the one described. In this case the object, after having been fixed and washed out, is taken from the water, or while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining, if that can be avoided), and passed through a bath of stain (generally alcoholic borax-carmine or other alcoholic stain) of sufficient duration, then dehydrated with successive alcohols, passed through a clearing medium into paraffin, cut, and treated as above described, the sections in this case being mounted direct from the turpentine, naphtha, or other solvent with which the paraffin is removed. If aqueous staining media be employed (and it is sometimes very desirable for particular purposes to prepare specimens with some aqueous stain) the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide, the objects being passed through successive baths of alcohol of gradually decreasing strength before being put into the aqueous stain (a precaution which will not be necessary for chromic objects (*see* below, § 5)).

It was stated in the first edition of this work that "the great majority of preparations are made by fixing either with

sublimate or a picric acid combination, washing out with alcohol, staining with alcoholic borax-carmine, imbedding in chloroform-paraffin, cutting with a sliding microtome, and mounting the sections in series in Canada balsam." That is probably still the case, but the method can no longer claim to be what it then appeared to be, the classical method of microscopic anatomy. I suggest the following, as being quite as easy to carry out, and as giving preparations far richer in detail and more truthfully preserved:—Fix in Flemming's chromo-aceto-osmic mixture; wash out with water; dehydrate; clear with oil of cedar-wood; imbed in paraffin; mount sections on the slide with Mayer's albumen medium; stain with safranin, or double-stain with gentian-violet and eosin; and mount in balsam or damar. That, or something like that, is now the practice of many of the most advanced workers; and I know of no method that seems to have equal claims to be considered a classical method of general morphological investigation.

As regards the method to be used for imbedding, I take it that the paraffin method is the method *par excellence* for small objects (objects up to 5 or 7 millimetres diameter); whilst the collodion or "celloïdin" method is the method *par excellence* for large objects.

As regards the rival claims of the method of staining objects *in toto* before section cutting, and that of staining the sections, I confess that I cannot see any reason for preferring the practice of staining *in toto*, which I consider only has a *raison d'être* in the case of objects which are not to be cut into sections.

3. The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to imbedding processes. Its normal course may be described as fixation, washing out, staining, treatment with successive alcohols of gradually increasing strength, final dehydration with absolute alcohol, clearing, and mounting in balsam. This method which may be termed the dehydration method, is usually preferred, as a general method, to what may be termed the wet methods, by which objects are prepared and preserved in aqueous media. The chief reason for this lies in the great superiority of the dehydration methods as regards the preservation of tissues. The presence of water is the most important factor in the conditions that bring about the

decomposition of organic matter, and its complete removal is the chief condition of permanent preservation.

It is of course not intended here to suggest that wet methods of preparation should be altogether discarded. They have great value, they are even indispensable, for special ends; and all that is intended to be suggested is, that they should be regarded not as *general* but as *Special* methods.

4. In the *preparation of entire objects* or structures that are intact and *covered by an integument not easily permeable* by liquids, special care must be taken to avoid swelling from endosmosis on the passage of the objects from any of the liquids employed to a liquid of less density, or shrinkage from exosmosis on the passage to a liquid of greater density. This applies most specially to the passage from the last alcohol into the clearing medium. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the clearing medium under the alcohol, as above described. Fluids of high diffusibility should be employed as far as possible in all the processes. Fixing agents of great penetrating power (such as picrosulphuric acid or alcoholic sublimate solution) should be employed where the objects present a not easily permeable integument. Washing out is done with successive alcohols, water being used only in the case of fixation by osmic acid, or the chromic mixtures or other fixing solutions that render washing by water imperative. Staining is done by preference with alcoholic staining media. The stains most to be recommended are Grenacher's borax-carmine, or one of Mayer's new carminic acid or hæmatein stains (for all of which *see* STAINING AGENTS). Anilin stains are rarely applicable to this class of preparations. Aqueous stains are more seldom used, though there are many cases in which they are admissible, and some in which they are preferable.

Minute dissections are best done, if necessary, in a drop of clearing agent. I recommend cedar-wood oil for this purpose, as it gives to the tissues a consistency very favorable for dissection, whilst its viscosity serves to lend support to delicate structures. Clove oil has a tendency to make tissues that have lain in it for some time very brittle. The brittleness is, however, sometimes very helpful in minute dissections. Another property of clove oil is that it does not easily spread

itself over the surface of a slide, but has a tendency to form very convex drops. This property also makes it frequently a very convenient medium for making minute dissections in.

5. Following Paul Mayer, I gave in the previous editions the following reasons for employing, in these cases, alcoholic rather than aqueous staining media. Since, in most cases, treatment with alcohol forms part of the fixing process, alcoholic solutions are logically indicated for staining. For by means of them it is possible to avoid the bad effects that follow on passing delicate tissues from alcohol into water, violent diffusive currents being thereby set up which sometimes carry away whole groups of cells; swellings being caused in the elements of the tissues; and, if the immersion in the aqueous medium be prolonged, as is generally necessary in order to obtain a thorough stain, maceration of the tissues supervening. But alcoholic staining fluids have still other advantages; they are in general more penetrating; with them alone is it possible to stain through chitinous integuments; and, if it be desired to stain slowly, tissues may be left in them for days without hurt.

Applied to the case now under consideration, the preparation *in toto* of objects protected by not easily permeable investments, this doctrine is evidently a wise one. For such objects must necessarily be fixed by some highly penetrating but not permanently hardening agent such as picric acid, and must necessarily be washed out with alcohol; and it is a good maxim for tissues so fixed that an object that has once been in alcohol should not be allowed to go back into water, if that can possibly be avoided.

But in the case of structures that have been well fixed in a strongly and permanently coagulating medium such as chromic acid, this precaution is much less necessary. Sections of tissues that have been fixed for twenty-four hours in Flemming's solution may be passed with relative impunity from absolute alcohol into an aqueous stain, and from that back again direct into absolute alcohol. It is this property of tissues fixed in chromic solution that determines me to recommend the practice of staining sections, instead of staining objects *in toto*. As regards the *quality* of the stain, aqueous solutions are in general the best. No alcoholic carmine or hematoxylin, for instance, will give a stain equal in precision and delicacy to those given by alum-carmine or Böhmer's hematoxylin. In a recent publication Mayer himself states that he has somewhat cooled in his preference for alcoholic staining media.

For an excellent exposition of the principles underlying the practice above recommended, the reader may consult with advantage the paper of Paul Mayer, in *Mitth. Zool. Stat. Neapel*, ii (1881), p. 1, *et seq.* See also the abstract in *Journ. Roy. Mic. Soc.* (N. S.), ii (1882), pp. 866—881, and that in *Amer. Natural*, xvi (1882), pp. 697—706, in which two last some improvements are mentioned which have been worked out since the publication of Mayer's paper.

CHAPTER II.

KILLING.

6. In the majority of cases, the first step in the preparation of an organ or organism consists in exposing it as rapidly and as completely as possible to the action of one of the **Fixing Agents** that are discussed in the next chapter. The organ or organism is thus taken in the normal living state; the fixing agent serves to bring about at the same time, and with sufficient rapidity, both the death of the organism and that of its histological elements.

But this method is by no means applicable to all cases. There are many animals, especially such as are of a soft consistence, and deprived of any rigid skeleton, but possessing a considerable faculty of contractility—such as many Cœlenterata, Bryozoa, and Serpulida, for instance—which if thus treated contract violently, draw in their tentacles or branchiæ, and die in a state of contraction that renders the preserved object a mere caricature of the living animal. In these cases, special methods of killing must be resorted to.

Sudden Killing.

7. Speaking generally, there are two ways of dealing with these difficult cases. You may kill the animal so suddenly that it has not time to contract; or you may paralyse it by narcotics before killing it.

The application of **Heat** is a good means of killing suddenly. It has the great advantage of allowing of good staining subsequently, and of hindering less than any other method the application of chemical tests to the tissues. By it, the tissues are fixed at the same time that somatic death is brought about. And what is more, the fixation thus brought about is extremely faithful, provided the operation be properly performed.

The difficulty consists in hitting off the right temperature, which is of course different for different objects. I think that a temperature of 80° to 90° C. will generally be amply sufficient, and that very frequently it will not be necessary to go beyond 60° C. An exposure to heat of a few seconds will generally suffice.

Small objects (Protozoa, Hydroids, Bryozoa) may be brought into a drop of water in a watch-glass or on a slide and heated over the flame of a spirit lamp. For large objects, the water or other liquid employed as the vehicle of the heat, may be heated beforehand and the animals thrown into it.

As soon as it is supposed that the protoplasm of the tissues is coagulated throughout, the animals should be brought into alcohol (30 to 70 per cent. alcohol) (if water be employed as the heating agent).

An excellent plan for preparing many marine animals is to kill them in *hot fresh* water. Some of the larger Nemertians, are better preserved by this method than by any other with which I am acquainted.

8. Animals that contract but slowly, such as Alcyonium and Veretillum, and some Tunicates, such as Pyrosoma, are very well killed by throwing them into some very quickly acting fixing liquid, either used hot or cold. *Glacial* or very strong *acetic acid* (**van Beneden's** method) is an excellent reagent for this purpose; it may be used, for example, with some Medusæ. After an immersion of a few seconds or a few minutes, according to the size of the animals, they should be brought into alcohol of at least 50 per cent. strength. See "**Acetic acid**" and "**Tunicata.**" *Corrosive sublimate* is another excellent reagent for this purpose.

Narcotisation.

9. The secret of narcotisation consists in adding some anæsthetic substance very gradually, in very small doses, to the water containing the animals, and waiting patiently for it to take effect slowly.

The Tobacco-smoke Method for Actiniæ (Hertwig, *Die Actinien*, 1879) used to be practised as follows:—A dish containing the animals in water is covered with a bell-glass, under which passes a curved glass or rubber tube, which dips into the water. Tobacco smoke is blown into the water for some time, through the tube, and the animals are then left for some hours in order that narcotisation may become fully established. The animals are irritated from time to time by touching a tentacle with a needle. As soon as it is observed that an animal begins to react slowly, that is to say as soon as it is found that the contraction of the tentacle does not begin

until a considerable time after it has been irritated by the needle, the narcotisation may be considered sufficient. A quantity of some fixing liquid sufficient to kill the animals before they have time to contract is then added to the water.

A space of several hours is necessary in order to thoroughly narcotise an Actinia by this method.

10. Nicotin in solution may be used instead of tobacco smoke (Andres, *Atti R. Accad. dei Lincei*, v. 1880, p. 9; see *Journ. Roy. Mic. Soc.*, N.S., ii, 1882, p. 881). Andres employs a solution of 1 gramme of nicotin in a litre of sea-water. The animal to be anæsthetised is placed in a jar containing half a litre of sea-water, and the solution of nicotin is gradually conducted into the jar by means of a thread acting as a siphon. The thread ought to be of such a thickness as to be capable of carrying over the whole of the solution of nicotin in twenty-four hours.

11. Chloroform may be employed either in the liquid state or in the state of vapour. Korotneff (*Mitth. Zool. Stat. Neapel*, v, Hft. 2, 1884, p. 229; *Zeit f. wiss. Mik.*, 2, 1885, p. 230) operates in the following manner with Siphonophora. The animals being extended, a watch-glass containing chloroform is floated on the surface of the water in which they are contained, and the whole is covered with a bell-glass. As soon as the animals have become insensible they are killed by means of hot sublimate or chromic-acid solution plentifully poured on to them.

Liquid chloroform is employed by squirting it in small quantities on to the surface of the water containing the animals. A syringe or pipette having a very small orifice, so as to thoroughly pulverise the chloroform, should be employed. Small quantities only should be projected at a time, and the dose should be repeated every five minutes, until the animals are anæsthetised.

I have seen large Medusæ very completely anæsthetised in the state of extension in an hour or two by this method. Andres finds that this plan does not succeed with Actiniæ, as with them maceration of the tissues supervenes before anæsthesia is established.

12. Ether and **Alcohol** may be administered in the same way. Andres has obtained good results with Actiniæ by the use of

a mixture (invented by Salvatore lo Bianco) containing 20 parts of glycerin, 40 parts of 70 per cent. alcohol, and 40 parts of sea-water. This mixture should be carefully poured on to the surface of the water containing the animals, and allowed to diffuse quietly through it. Several hours are sometimes necessary for this.

Eisig employs alcohol in the same way.

13. Methyl-alcohol.—Cori (*Zeit. f. wiss. Mik.*, vi, 4, 1890, p. 438) prefers methyl-alcohol to all other reagents. It has, amongst other advantages, that of having but a slight action on albumins. Cori recommends a mixture composed of 10 c.c. methyl-alcohol (of 96 per cent. strength), 90 c.c. water (fresh or sea-water), and 0·6 g. of sodium chloride (to be added only when fresh water is taken, the addition of the salt having for its object to prevent maceration).

14. Hydrate of Chloral, which was first recommended, I belive, by Foettinger (*Arch. de Biol.*, vi, 1885, p. 115), gives very good results with some subjects. Foettinger operates by dropping crystals of chloral into the water containing the animals. For Alcyonella he takes 25 to 80 centigrammes of chloral for each hundred grammes of water. It takes about three quarters of an hour to render a colony sufficiently insensible to allow of fixing. Foettinger has obtained satisfactory results with marine and fresh-water Bryozoa, with Annelida, Mollusca, Nemertians, Actiniæ, and with *Asteracanthion*. He did not succeed with Hydroids.

I am bound to state that I have never had the slightest success with Nemertians.

Verworn (*Zeit. f. wiss. Zool.*, xlvi, 9, 1887, p. 99; see also *Journ. Roy Mic. Soc.*, 1888, p. 148) operates differently for fresh-water Bryozoa. He puts *Cristatella* for a few minutes into 10 per cent. solution of chloral, in which the animals sooner or later become extended.

Kükenthal (*Zeit. f. wiss. Mik.*, iv, 8, 1887, p. 878; *Journ. Roy. Mic. Soc.*, 1888, p. 509) has obtained good results with some Annelids, by means of a solution of one part of chloral in 1000 parts of sea-water.

The chloral method gives rise to maceration with some subjects, and has been found to distort nuclear figures. (This, I suspect, will probably be found to be the case with most of these methods.)

15. Cocaïn (Richard; *Zool. Anz.*, 196, 1885, p. 332) has been found to give good results. Richard puts a colony of Bryozoa into a watch-glass with 5 c.c. of water, and adds gradually 1 per cent. solution of hydrochlorate of cocaïn in water. After five minutes, the animals are somewhat numbed, and half a cubic centimetre of the solution is added, and the tentacles are caused to contract by irritating them with a needle. Ten minutes later the animals should be found to be dead in a state of extension.

This method is stated to succeed with Bryozoa, *Hydra*, and certain worms.

It has been pointed out (by Cori, in the paper quoted above) that unfortunately when fixing agents, such as sublimate solution, are added to the animals, the cocaïn is thrown down on them as a white precipitate.

16. Hydroxylamin.—Hofer (*Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 318) has employed hydroxylamin as a paralysing agent with success with the most varied animal forms. Either the sulphate or the hydrochlorate of the base may be used. He recommends that the hydrochlorate be taken. This, as found in commerce, is usually contaminated with HCl. It should be dissolved in water (spring or sea-water, according to the habitat of the organisms—in no case distilled water) and the solution exactly neutralised by addition of carbonate of soda. A 1 per cent. solution should be made up, and further diluted for use. The organisms are placed in the diluted solution, which may be taken of a strength varying from 0·1 per cent, used for thirty minutes or less (as for Infusoria) to 0·25 per cent., used for from fifteen minutes to one hour (*Hydra*), 1 per cent., one half to two hours (*Hirudo*), or as much as ten to twenty hours (*Helix* and *Anodonta*).

It should be remembered that hydroxylamin is an extremely powerful reducing agent. Care must therefore be taken not to treat the paralysed animals with easily reducible fixing agents, such as osmic acid, chromic acid, sublimate, chlorides of gold or platinum, &c., unless it have been possible first to sufficiently wash out the hydroxylamin with water.

17. Chloride of Magnesium—Tullberg (*Arch. Zool. Expér. et Gén.*, x, 1892, p. 11; *Journ. Roy. Mic. Soc.*, 1892, p. 435) has obtained some results with this salt. For Actiniæ, a 33 per cent. solution of the salt is to be very slowly added to the water containing the expanded animal, until the vessel contains one per cent. of the salt (thus for one litre of sea-water

33 c.c. of the solution must be added). The addition must be made gradually; but it must be effected within half an hour. Thirty minutes later the animal will be found to be anæsthetised, and may be fixed.

For terrestrial and fresh-water Invertebrates, rather stronger solutions should be used.

18. Poisoning by small doses of some fixing agent is sometimes a good method. SALVATORE LO BIANCO employs the following method for preserving Ascidiæ in an extended state. A 1 per cent. solution of chromic acid acidulated with acetic acid is poured on to the surface of the water containing the animals, and allowed to diffuse slowly through it. The operation takes four or five days (v. GARBINI, *Manuale per la Technica Mod. del Microscopio*, p. 168).

Osmic acid, or Kleinenberg's solution, is sometimes employed in the same way.

I have seen Medusæ killed in a satisfactory manner by means of crystals of corrosive sublimate added to the water containing them.

Morphia, Curare, Strychnin, Prussic Acid, and other paralysing drugs have also been employed.

19. Asphyxiation may be sometimes successfully practised. Terrestrial Gastropods may be killed for dissection by putting them into a jar quite full of water that has been deprived of its air by boiling, and hermetically closed. After from twelve to twenty-four hours the animals are generally found dead and extended. The effect is obtained somewhat quicker if a little tobacco be added to the water.

Good results are sometimes obtained with aquatic animals by simply leaving them to exhaust the oxygen of the water in which they are contained. I have sometimes succeeded with Holothuriæ and other Echinoderms in this way; and WARD (see *Amer. Nat.*, XXV, 1891, p. 398) has succeeded with Hydroids, Actiniæ, and similar forms. If the animals be found to be imperfectly expanded when narcosis has set in, they may be got to expand by putting them back for a short time into pure sea-water; and as soon as they are expanded should be quickly thrown into some rapidly-killing reagent.

Carbonic Acid Gas has been recommended (by FOL, *Zool. Anz.*, 128, 1885, p. 698). The water containing the animals should be saturated with the gas. The method is stated to succeed with most Cœlenterata and Echinodermata, but not with Molluscs or Fishes. It has not been found successful at the Naples laboratory.

20. Marine Animals are sometimes successfully killed by simply putting them into fresh water.

Warm Water will sometimes serve to immobilise and even kill both marine and fresh-water organisms.

CHAPTER III.

FIXING.

21. The Necessity of Fixing.—The meaning of the term "fixing" has been explained above (§ 2). It only remains here to insist on the absolute necessity of the employment of fixing agents, and to briefly illustrate this necessity by a single example. If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer segments of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

22. The Action of Fixing Agents consists in the coagulation of certain of the constituents of tissues, of their albuminoids, their gelatin, their mucin. Some fixing agents seem to have further the property of combining chemically with the tissues,

so that they cannot be easily removed from them by washing. This is a consideration of great importance in view of ulterior operations, and most particularly in view of staining. Chromic acid and its salts, osmic acid, the chlorides of palladium, of gold, and of iron, are reagents that seem to combine chemically with the tissues, and render necessary a special after-treatment and special modes of staining, whilst picric acid, nitric acid, and corrosive sublimate do not appear to enter into that kind of combination, and can be entirely removed from the tissues by washing, and leave the tissues in a state in which they are susceptible of any kind of staining.

Practically it amounts to this, that if you fix with a chromic or osmic mixture, you cannot stain with carmine but can only stain with hæmatoxylin, if you wish to stain your objects *in toto;* or you may make sections, and stain them with safranin or some other coal-tar colour. Whilst if you fix with sublimate or a picric acid mixture, you may do as you like in the matter of staining.

The after-treatment appropriate to each fixing agent is indicated in the special paragraphs.

23. Choice of a Fixing Agent.—Indications concerning the proper fixing agent to employ for the different tissues and organs of the animal kingdom, will be found in Part II. The following remarks are intended as hints for beginners only.

The chief fixing agents for general work are FLEMMING'S *mixture*, HERMANN'S *platinum-chloride mixture*, osmic acid, corrosive sublimate, and picro-sulphuric acid.

I recommend that Flemming's mixture should be used wherever it is possible, as I believe it to be in general by far the best fixing agent yet invented, with the exception of Hermann's mixture, which is unfortunately too expensive to be employed in large quantities.

But it will not always be found possible to use it. Its low power of penetration, for instance, puts it out of court in the case of very impermeable objects, such as are frequently offered by the Arthropoda. For these, picro-sulphuric acid may be recommended.

For very small objects, such as may be mounted whole, osmic acid is nearly as good as Flemming's mixture, and is frequently much more convenient to use.

For objects a little larger, and for much embryological work on objects that it is convenient to have stained *in toto*, corrosive sublimate may be recommended. I think it is in general a much better preservative of the forms of anatomical elements than either Altmann's nitric acid or Kleinenberg's fluid.

24. The Practice of Fixation,—Hints and Cautions.—See that the structures are *perfectly living* at the instant of fixation, otherwise you will only fix pathological states or post-mortem states.

Do all you can to facilitate the *rapid penetration* of the fixing agent. To this end, let the structures be divided into the smallest portions that can conveniently be employed, and if entire organs or organisms are to be fixed whole, let openings, as large as possible, be first made in them.

The penetration of reagents is greatly facilitated by *heat*. You may warm the reagent and put it with the objects to be fixed in the paraffin stove, or you may even employ a fixing agent heated to boiling point (as boiling sublimate solution for certain corals and Hydroids, or boiling absolute alcohol for certain Arthropods with very resistant integuments).

Let the *quantity* of fixing agent employed be at least *many times* the volume of the objects to be fixed. If this precaution be not observed the composition of the fixing liquid may be seriously altered by admixture of the liquids or of the soluble substances of the tissues thrown into it. For a weak and slowly acting fixing agent, such as picric acid, the quantity of liquid employed should be in volume about one hundred times that of the object to be fixed. Reagents that act very energetically, such as Flemming's solution, may be employed in smaller proportions.

Be careful to use the *appropriate liquid for washing out* the fixing agent after fixation. It is frequently by no means a matter of indifference whether water or alcohol be employed for washing out. Sometimes water will undo the whole work of fixation (as with picric acid). Sometimes alcohol causes precipitates that may ruin the preparations. Instructions on this head are given in the paragraphs devoted to the different fixing agents.

Use *liberal quantities* of liquid for washing.

Change the liquid as often as it becomes turbid, if that should happen.

The process of washing out is often greatly facilitated by *heat*. Picric acid, for instance, is nearly twice as soluble in alcohol warmed to 40° C. as in alcohol at the normal temperature (Fol).

In the case of *marine organisms* it may be stated as a general rule that their tissues are more refractory to the action of reagents than are the tissues of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times stronger, according to Langerhans).

Marine animals ought to be *freed from the sea-water* adherent to their surfaces before treating them either with alcohol or any fixing reagent that precipitates the salts of sea-water. If this be not done, the precipitated salts will form on the surfaces of the organisms a crust that prevents the penetration of reagents to the interior, thus allowing maceration to be set up, and hindering the penetration of staining fluids. Fixing solutions for marine organisms should therefore be such as serve to keep in a state of solution, and finally remove, the salts in question. They should *never be made with sea-water* as a menstruum, as some workers have inconsiderately proposed. If alcohol be employed it should be acidified with hydrochloric acid. Picro-sulphuric acid is a reagent that fulfils the conditions here spoken of. (On this subject see Paul Mayer, in *Mitth. Zool. Stat. Neapel,* ii (1881), p. 1 et seq. See also the abstract in *Journ. Roy. Mic. Soc.* (N.S.), ii (1882), pp. 866—881, and that in *Amer. Natural.*, xvi (1882), pp. 697—706.)

CHAPTER IV.

FIXING AGENTS. MINERAL ACIDS AND THEIR SALTS.

25. Osmic Acid.—The tetroxyde of osmium (OsO_4) is the substance commonly known as osmic acid, though it does not possess acid properties. It is a substance that is exceedingly difficult to keep in use for any length of time. It is extremely volatile, and in the form of an aqueous solution becomes partially reduced with great readiness in presence of the slightest contaminating particle of organic matter. (It is generally believed that the aqueous solutions are reduced by light alone, but this is not the case; they may be exposed to the light with impunity if dust be absolutely denied access to them. It would even seem that the solutions are improved for some purposes by exposure to sunlight, *vide infra*, the remarks on gold chloride solutions. Some observations communicated to me by Dr. Lindsay-Johnson go to prove that, if dust be avoided, solutions keep better in the light, with occasional "sunning," than in the dark.)

Great stress is laid by authors on the fact that the vapour of osmium is very irritating to mucous tissues. It is said that the slightest exposure to it is sufficient to give rise to serious catarrh, irritation of the bronchial tubes, laryngeal catarrh, conjunctivitis, &c. I have never myself suffered in this way, but there is no doubt that many persons do, and such susceptible subjects should be very careful in handling osmium in any form.

26. How to keep the Solutions.—After having carefully tried several of the plans that have been recommended for keeping the working solutions free from dust, I have come to the conclusion that the following is the most practical plan for preventing them from "going bad":—The solution of osmic acid in chromic acid solution is not, like the solution in pure water, easily reducible, but may be kept without any special precautions. I therefore keep the bulk of my osmium in the shape of a 2 per cent. solution of osmic acid in 1 per cent. aqueous chromic acid solution. This solution serves for

fixation by osmium vapours, and for making up solution of Flemming (or solution of Hermann) which is the form in which osmium is most generally employed. A small quantity of osmic acid may also be made up in 1 per cent. solution in distilled water, and kept carefully protected from dust for use in special cases. Those who have to do a great deal of fixing by means of the vapours may also keep a supply of the solid oxide for this purpose.

Cori (*Zeit. f. wiss. Mik.*, vi, 4, 1890, p. 442) finds that solutions in distilled water keep perfectly if there be added to them enough permanganate of potassium to give a very slight rosy tint to the liquid. From time to time, as the solution becomes colourless, further small quantities of the salt should be added, so as to keep up the rosy tint.

27. Fixation by the Vapours.—Osmic acid is frequently employed in the form of vapour, and its employment in this form is indicated in most of the cases in which it is possible to expose the tissues directly to the action of the vapour. The tissues are pinned out on a cork which must fit well into a wide-mouthed bottle in which is contained a little solid osmic acid (or a small quantity of 1 per cent. solution will do). Very small objects, such as isolated cells, are simply placed on a slide, which is inverted over the mouth of the bottle. They remain there until they begin to turn brown (isolated cells will generally be found to be sufficiently fixed in thirty seconds, whilst in order to fix the deeper layers of relatively thick objects, such as retina, an exposure of several hours may be desirable). It is well to wash the objects with water before staining, but a very slight washing will suffice. For staining, methyl-green may be recommended for objects destined for study in an aqueous medium, and, for permanent preparations, alum-carmine, picro-carmine, or hæmatoxylin.

In researches on nuclei, it is possible and may be useful to employ the vapours of a freshly-prepared mixture of osmic and formic or acetic acid (Gilson, *La Cellule*, i, 1885, p. 96).

The reasons for preferring the process of fixation by vapour of osmium, where practicable, are that osmium is more highly penetrating when employed in this shape than when employed in solution, and produces a more *equal* fixation, and that the arduous washing out required by the solutions is here done away with. In many cases delicate structures are better preserved, all possibility of deformation through osmosis being here eliminated.

28. Fixation by Solutions.—When employed in aqueous solutions osmic acid is used in strengths varying from $\frac{1}{20}$ per cent. to 2 per cent. Solutions of $\frac{1}{2}$ per cent. to 1 per cent. have been very largely used, but the tendency of modern practice seems to be towards weaker solutions and longer immersion. For Infusoria $\frac{1}{2}$ per cent. for a few seconds; for Porifera $\frac{1}{20}$ to $\frac{1}{10}$ per cent. for some hours; for Mollusca 1 to 2 per cent. for twenty-four hours; for epithelia $\frac{1}{10}$ to $\frac{1}{2}$ per cent. for an hour or two; for meroblastic ova $\frac{1}{10}$ per cent. for twenty-four hours; for medullated nerve-fibre $\frac{1}{10}$ to 1 per cent. for from twenty minutes to two hours; for tactile corpuscles $\frac{1}{3}$ to 1 per cent. for twenty-four hours; for retina $\frac{1}{4}$ to 2 per cent. for from ten minutes to twenty-four hours; for nuclei $\frac{1}{10}$ to 2 per cent. for two or three hours. Such figures as these will serve to give a general idea of the practice, whilst more precise instructions will be given when dealing with the tissues in detail. (The durations here quoted appear to me exaggerated, except for very voluminous specimens.)

A little acetic or formic acid (0·5 to 1 per cent.) may frequently with advantage be added to the solutions just before using.

If solutions made with pure water be used, they must be kept protected from the light during the immersion of tissues. This precaution is not necessary if Flemming's or Hermann's solution be used. If the immersion is to be a long one the tissues must be placed with the solution in well-closed vessels, as osmium is very volatile.

Kolossow (*Zeit. f. wiss. Mik.*, v, i, 1888, p. 51) has recommended a 0·5 per cent. solution of osmium in 2 or 3 per cent. solution of nitrate or acetate of uranium, as having a greatly enhanced penetrating power.

He has more lately (op. cit., ix, 1, 1892, p. 39) recommended for the same reason a mixture of 50 c.c. absolute alcohol, 50 c.c. distilled water, 2 c.c. concentrated nitric acid, and 1 to 2 g. osmium. This mixture is said to keep indefinitely in a cool place.

The principle of combining osmium solutions with alcohol is due to Ranvier et Vignal (Ranvier, *Leç. d'Anat. Gén.*, "App. term. des muscles de la vie org.," p. 76; Vignal, *Arch. de Physiol.*, 1884, p. 181). They take equal volumes of 1 per cent. osmic acid and 90 per cent. alcohol (freshly mixed). They wash out in 80 per cent. alcohol, then wash with water and stain for forty-eight hours in picro-carmine or hæmatoxylin. Viallanes has applied this method to the histology of insects.

29. After-treatment.—The osmium must be well washed out

before proceeding to any further steps in preparation; water should be used for washing. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to over-blacken in time. To obviate this it is necessary to wash them out in ammonia-carmine or picro-carmine, or to soak them for twenty-four hours in a solution of bichromate of potash (Müller's solution or Erlicki's will do), or in 0·5 per cent. solution of chromic acid, or in Merkel's solution, or in a weak solution of ferrocyanide of potassium or cyanide of potassium. The treatment with bichromate solutions has the great advantage of highly facilitating staining with carmine or hæmatoxylin. Max Schultze recommended washing, and mounting permanently in acetate of potash; but I believe the virtues attributed to this method are illusory. Fol has lately recommended treatment with a weak solution of carbonate of ammonia. But the best plan of all is to properly *bleach* the preparations (see "**Bleaching**"). This is perhaps most coveniently done (as recommended by Fol, Brass, and Overton) by means of *peroxide of hydrogen*, which regenerates the osmium to osmic acid. Overton (*Zeit. f. wiss. Mik.*, vii, 1, 1890) finds that bleaching is completed in a few minutes in a mixture of 1 part commercial peroxide of hydrogen with 10 to 25 parts 70 per cent. alcohol. (The commercial peroxide, slightly acidulated with HCl, will keep well in the dark; but the mixture with alcohol must be made fresh for use.)

The same stains recommended for objects fixed by vapour will be found useful here, with the addition of ammonia-carmine, which is really very useful for strongly fixed specimens. For sections, of course in both cases, safranin and the other nuclear anilin stains may be employed with advantage.

Osmic acid stains all fatty structures black; it must therefore be avoided for tissues in which much fat is present; or if not, the preparations must be subsequently very thoroughly bleached.

30. Chromic Acid.—Chromic anhydride, CrO_3, is found in commerce in the form of red crystals that dissolve readily in water, forming chromic acid, H_2CrO_4. These crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution. Care must be taken not to allow the crystals to be contaminated by organic matter, in the presence of which the anhydride is readily reduced into sesquioxide.

Chromic acid is employed in solution either in water or in alcohol.

The most useful strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve-tissues weaker solutions are taken, $\frac{1}{50}$th to $\frac{1}{8}$th per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

The object should be washed out with water before passing into alcohol or staining fluids. Long washing in water is necessary to prepare them for staining, except an anilin stain be used. It is possible to wash out in alcohol, and this may be useful in special cases, but in general I think the practice is not to be recommended. It is well to wash for many hours in *running* water.

Tissues that have been fixed in chromic acid may be stained in aqueous solutions if desired, as water does not appear to have an injurious effect on them; the acid appears to enter into some chemical combination with the elements of the tissues, forming with them a compound that is not affected either physically or chemically by water. The best stain to follow chromic acid is hæmatoxylin, or, for sections, some anilin stain. But the previous washing out with water must be very thorough if good results are to be insured; it may take days.

Chromic acid is not a very penetrating reagent, and for this reason, as well as for others, is seldem used pure, but plays an important part in the mixtures described below, of which the chief is certainly the mixture of Flemming. A chief objection to the use of chromic acid is that *it precipitates certain of the liquid albuminoids of tissues in the form of filaments or networks,* which are often of great regularity, and *simulate structural elements of the tissues.* This objection applies to all mixtures into which chromic acid enters.

Action of light on alcohol containing chromic objects.—When objects that have been treated by chromic acid or a chromate are put into alcohol for hardening or preservation, it is found that after a short time a fine precipitate is thrown down on the surface of the preparations, thus forming a certain obstacle to the further penetration of the alcohol. Previous washing by water does not prevent the formation of this precipitate, and changing the alcohol does not prevent it from forming again and again. It has been found by Hans Virchow (*Arch. f. mik. Anat.*, Bd. xxiv, 1885, p. 117) that

the formation of this precipitate may be entirely prevented by simply keeping the preparations in the dark. The alcohol becomes yellow as usual (and should be changed as often as this takes place), but no precipitate is formed. If this precaution be taken, previous washing with water may be omitted, or at all events greatly abridged.

The brownish-green colour of chromic objects may be removed by treating them with peroxide of hydrogen (Unna, in *Arch. f. mik. Anat.*, Bd. xxx, 1887, p. 47; cf. *Journ. Roy. Mic. Soc.*, 1887, p. 1060; and see the instructions for bleaching osmic acid preparations at the end of the last §).

31. Chromo-acetic Acid (Flemming, *Zellsbz. Kern. u. Zellth.*, p. 382).

Chromic acid .	0·2 to 0·25 per cent.
Acetic acid . .	0·1 per cent., in water.

Flemming finds this the best reagent for the study of the *achromatic* elements of karyokinesis. (Flemming wrote this in 1882, and I doubt whether it would now hold good.) Stain with hæmatoxylin (the preparations are *not* favourable for staining with safranin or other coal-tar colours).

I can recommend as a good fixing and hardening mixture for Annelids in general, and probably for other forms, the following fluid due to Ehlers (I do not know whether it has been published):—To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The proportion of acetic acid indicated is sufficient to counteract any tendency to shrinkage due to the chromic acid.

32. Chromo-formic Acid (Rabl, *Morph. Jahrb.*, x, 1884, pp. 215, 216).—Four or five drops of concentrated formic acid are added to 200 c.c. of 0·33 per cent. chromic acid solution. The mixture must be freshly prepared at the instant of using. Fix for twelve to twenty-four hours, wash out with water, harden in alcohol, stain with hæmatoxylin or safranin. For the study of karyokinesis. This is acknowledged to be one of the very best reagents for the purpose.

33. Chromic Acid and Spirit.—A mixture of 2 parts of $\frac{1}{6}$th per cent. chromic acid solution with 1 part of methylated spirit was much used by Klein in his investigations into the structure of cells and nuclei, and found to give better results than the ordinary reagents (including even osmic acid). Hæmatoxylin was used for staining.

The addition of alcohol to augment the penetrating power of chromic acid seems to be a step in the right direction, and

it is matter for surprise that such mixtures are not more used. The alcohol should be added to the acid in aqueous solution, as if strong alcohol be added to crystals of chromic anhydride, a very violent reaction is set up. The mixture should be kept in the dark.

34. Chromo-osmic Acid (MAX FLESCH, *Arch. f. mik. Anat.*, xvi, 1879, p. 300).—This mixture (osmium 0·10, chromic acid 0.25, water 100·0), originally introduced for the preparation of the auditory organ of vertebrates, is of general application. It does not require to be kept in the dark. Objects may remain in it for twenty-four or thirty-six hours without risk of the osmic acid over-blackening them. Flemming found it to preserve nuclear figures well, but the preparations are pale, and difficult to stain well. He finds that the action of the mixture is improved (for nuclear figures) by the addition of acetic, formic, or other acid. This addition brings out the figures more sharply, and has the further advantage of allowing of a sharper stain with hæmatoxylin, picro-carmine, or gentian violet. He recommends the following formula, which may be considered to have superseded Max Flesch's.

35. Chromic-aceto-osmic Acid (FLEMMING, FIRST OR WEAK formula, *Zellsubstanz, Kern und Zelltheilung*, 1882, p. 381).—

Chromic acid . .	0·25 per cent.	In water.
Osmic acid . .	0·1 per cent.	
Glacial acetic acid .	0·1 per cent.	

The best results (as regards faithfulness of fixation) are obtained with this mixture when it is allowed to act for only a short time (about half an hour).

But it may, without inconvenience, be allowed to act for many hours or even days. Wash out, very thoroughly, in water. Stain with hæmatoxylin, if you wish to stain *in toto* (staining in this way with other reagents is possible, but very difficult, and not to be recommended). Stain sections with safranin, or other anilin, or with hæmatoxylin or Kernschwarz.

To make up this mixture with the usual stock solutions, you take:

Chromic acid of 1 per cent. . .	25 volumes.
Osmic acid of 1 per cent. . .	10 ,,
Acetic acid of 1 per cent. . .	10 ,,
Water	55 ,,

If you keep your osmium in 2 per cent. solution in chromic acid of 1 per cent., as I have recommended, you will have to take only 20 vols. of chromic acid, 5 of your osmium solution,

and 65 of water. See also the remarks on the deterioration of these solutions by keeping, in the next §.

It has been already stated more than once that Flemming's solution is, with the exception of Hermann's solution, probably the very best fixing reagent in general yet discovered. It has, however, been criticised. Faussek (*Zeitschr. f. wiss. Zool.*, Bd. xlv, 1887, pp. 694, *et seq.*) found it totally inapplicable to the histology of the intestine of insects. He states that it caused the intima to disappear, and the cells to run together into a compact mass. Arnold (*Arch. f. mik. Anat.*, Bd. xxx, 1887, p. 205) states that it does not preserve cell-bodies faithfully. And A. Kotlarewsky (*Mitth. d. naturf. Ges. Bern.*, 1887; cf. *Zeit, f. wiss. Mik.*, iv, 3, 1887, p. 387) found that it preserved the forms of nerve-cells (spinal ganglia) less faithfully than any of the reagents tried. I have not, myself, been struck by any decided defect in the preservation of cystoplasmic structures in my preparations made by this reagent, but think it possible that the observations of these authors may be well founded as regards the present formula, but take it that that is merely a reason for preferring the stronger mixture set forth below.

It is not necessary in all cases to observe the exact proportions of the ingredients in this mixture. Fol (*Lehrb. d. vergl. mik. Anat.*, 1884, p. 100) recommends the following:

1 per cent. chromic acid	25 vols.
1 per cent. osmic acid	2 „
2 per cent. acetic acid	5 „
Water	68 „

That is to say, a mixture much weaker in osmium than Flemming's. In the *Traité des Méthodes Techniques*, &c., Lee et Henneguy, 1887, I recommended this mixture, as giving better results in general, but am now inclined to think that, at all events as regards fidelity of fixation, it is a step in the wrong direction, and that, on the contrary, the stronger mixture of Flemming (next §) is a step in the right direction. Fol's formula has the advantage of allowing better staining with carmine, that is all.

A mixture still weaker than this in osmium, viz. with 1 vol. osmium solution instead of 2, has been recommended by Cori (*Zeit. f. wiss. Mik.*, vi, 1, 1890, p. 441), but I still adhere to the opinion above expressed.

36. Chromo-aceto-osmic Acid (Flemming, second or strong formula, *Zeit. f. wiss. Mik.*, 1, 1884, p. 349).—

1 per cent. chromic acid	15 parts.
2 per cent. osmic acid	4 „
Glacial acetic acid	1 „

If 2 per cent. osmium solution should not be at hand, you may conveniently make the mixture by taking

10 per cent. chromic acid	15 parts.
1 per cent. osmic acid	80 „
Glacial acetic acid	10 „
Water	95 „

If this mixture be kept in stock in large quantities, it may go bad, probably on account of the large proportion of organic acid contained in it. I therefore recommend that it be made up from time to time from stock solutions, in which the osmium is kept separate from the acetic acid. The proportions being as follows:

CrO_3	0·15
Os.	0·08
Acid. acet.	1·00
Aq.	19·00

You may make up and keep separately—

(A) 1 per cent. chromic acid . .	11 parts.
Distilled water	4 „
Glacial acetic acid . . .	1 „

and (B) a 2 per cent. solution of osmium in 1 per cent. chromic acid solution, and when required, mix four parts of A with one of B; or, of course, if you prefer it, you may keep the osmic and chromic acid ready mixed in the proportions given, and add 5 per cent. of acetic acid at the moment of using.

In any case, it is better not to make up very large quantities of the mixture at once, as osmium being very volatile it will be found that solutions that have been long in use no longer contain the proper proportion of that ingredient, and the hardening action being thus weakened the swelling action of the acetic acid will be insufficiently controlled.

Merk (*Denksch. d. Math. Naturw. Cl. d. K. Acad. d. Wiss. Wien*, 1887; cf. *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 237) proposes to make up separately (A)

2 per cent. chromic acid	7·5 parts.
Water	3·5 „
Acetic acid	1 „

and (B), some 1 per cent. osmium solution. and to mix for use 12 parts of A with 8 of B. But this plan leaves you in the old difficulty of keeping your osmium in aqueous solution.

It does not appear necessary to observe the exact proportions of the ingredients of these mixtures, a certain latitude is allowable. Thus Carnoy (*La Cellule*, 1, 2, 1885, p. 211) has employed a mixture one third stronger in osmium and twice as strong in chromic acid, viz.

Chromic acid of 2 per cent. (or even stronger) .	45 parts.
Osmic acid of 2 per cent.	16 „
Glacial acetic acid	3 „

In the *Traité des Méth. Techniques*, 1887, I treated this

formula somewhat coldly, pointing out (what was the case) that Flemming recommended it merely for a very special purpose, the hunting for karyokinetic figures, and that he did not recommend it for general purposes. Further experience has shown that it is applicable to general purposes, and will probably be found for most purposes considerably superior to the weak formula. I should use it by the hogshead if it were not somewhat expensive.'

Arnold, in the place quoted in the last paragraph, says that it is to be avoided if you wish to demonstrate the structure of certain nuclei (of wandering cells); and the other objections there quoted as applying to the weak formula are intended to apply more or less to the present formula. It will be well not to attach too much importance to them. Let delicate structures be fixed for twenty-four hours or more, washed in running water for an hour, and in successive alcohols for twenty-four hours, sectioned, and stained with safranin or gentian violet. and there will be little complaint of defective preservation.

The strong mixture does not brown tissues more than the weak mixture, but rather less.

Fat is blackened by these mixtures; but the blackened fat can be entirely dissolved out of the tissues by treating them for a few hours with turpentine that has been exposed to sunlight for an hour or two (*see* Flemming in *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 39; and vi, 2, 1889. p. 178).

Podwyssozki recommends (for glands especially) the following modification:

1 per cent. $Cro._3$ dissolved in 0·5 per cent. solution of corrosive sublimate	15 c.c.
2 per cent. osmium solution	4 c.c.
Glacial acetic acid	6 to 8 drops.

This sublimate is said to augment the penetration of the osmium, but is unfavorable to staining. The proportion of acetic acid is reduced in order to avoid swelling of the tissue-elements (Ziegler's *Beitrage z. path. Anat.*, i, 1886; cf. *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 405).

36a. Platino-aceto-osmic Acid (Hermann's **solution**). This *extremely important* reagent is historically a modification of Flemming's solution, platinum-chloride being taken instead of chromic acid. See next Chapter, § 51.

37. Nitric Acid (Altmann, *Arch. Anat. u. Phys.*, 1881, p. 219).

Altmann employs dilute nitric acid, containing from 3 to 3½ per cent. pure acid. Such a solution has a sp. gr. of about 1·02; an aræometer may conveniently be used to determine the concentration of the solution. Stronger solutions have been used, but do not give such good final results.

His (*ibid.*, 1877, p. 115) recommended a 10 per cent. solution. Flemming

at one time employed solutions of 40 to 50 per cent. for the ova of invertebrates. This of course has the advantage of a very rapid fixing action.

Nitric acid has the valuable property of hardening yolk without making it brittle. But, for general purposes at all events, the pure nitric acid solutions may be considered to be superseded by Perenyi's chromo-nitric acid mixture (below, § 39).

38. Nitrate of Silver has been used for fixation, but is too uncertain in its action to be recommendable.

39. Chromo-nitric Acid (PERENYI'S formula, *Zool. Anzeig.*, v, 1882, p. 459).

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

These are mixed, and after a short time give a fine violet-coloured solution.

The objects are immersed for four to five hours, and then passed through 70 per cent. alcohol (twenty-four hours) strong alcohol (some days), absolute alcohol (four to five days). They are then fit for cutting. The advantage of the process is, amongst others, that segmentation spheres and nuclei are perfectly fixed, the ova do not become porous, and cut like cartilage.

Chromo-nitric acid is not only an embryological reagent, and a very important one, but also an admirable one for general work. I have found it altogether excellent for preserving marine organisms, especially large forms. Strong alcohol need only be used if the objects are destined to be sectioned.

Another advantage is that the fixing solution may be combined with a stain. (In this case the albuminous envelopes of the ova must be carefully removed, otherwise the stain will not penetrate.)

Some stains, such as fuchsin or anilin red, may be dissolved directly in the fixing solution. Others, such as eosin, purpurin, anilin violet, must first be "dissolved in three parts of alcohol, and then shaken into the liquid."

Picro-carmine and borax-carmine may be added to the liquid, but they give rise to a precipitate, which must be removed by filtration before using.

Another formula given by Perenyi (*Zool. Anzeig.*, 274, 1888, p. 139, and 276, p. 196) is as follows:

3 parts 20 per cent. nitric acid.
3 parts 1 per cent. chromic acid.
4 parts absolute alcohol.

For embryos of Lacerta. Fix for twenty minutes. Wash out for an hour with 70 per cent. alcohol, and then with strong alcohol. Stain with Dela-

field's hæmatoxylin, and treat the stained material for three to five minutes with 1 per cent. chromic acid.

40. Chromo-nitric Acid with Bichromate (KOLLMANN, *Arch. f. Anat. u. Phys.*, 1885, p. 296).

Bichromate of potash	5 per 100.
Chromic acid	2 „
Concentrated nitric acid	2 „

For ova of Teleostea. Fix for twelve hours, wash with water for twelve hours, then remove the chorion, and put the ova into 70 per cent. alcohol.

41. Picro-chromic Acid (*Fol. Lehrb.*, p. 100).—

Picric acid, sol. sat. in water	10 vols.
1 per cent. chromic acid solution . . .	25 „
Water	65 „

At the instant of using, you may add 0·005 of osmic acid, which makes the action more energetic. Wash with water (hot, nearly boiling water is best), and then with alcohol. Fol says, "This reagent hardens tissues admirably, without hindering staining in any way; but it is not very penetrating and fixes slowly."

I have seen Fol's formula, with the addition of a trace of acetic acid quoted as "liquid of Hænsel"—I know not with what justification.

42. Chromic Acid and Platinic Chloride (*Merkel's solution*; from *Mitth. Zool. Stat. Neapel*, 1881, p. 11).—Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinic chloride (PtCl 4). Objects should remain in it for several hours or even days, as it does not harden very rapidly. After washing out with alcohol of 50 per cent. to 70 per cent., objects stain excellently, notwithstanding the admixture of chromic acid. This is a very delicate and admirable fixative. If objects that have been fixed by osmium be put into it for some hours, blackening is effectually prevented. See below § 50.

Salts.

43. Bichromate and Cupric Sulphate Mixture (KULTSCHITZKY, *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 348).—A saturated solution of bichromate of potash and sulphate of copper in 50 per cent. alcohol, to which is added at the instant of using a little acetic acid, five or six drops per 100 c.c.

To make the solution, add the finely powdered salts to the alcohol in excess, and leave them together *in total darkness*, for twenty-four hours.

Fix for twelve to twenty-four hours *in the dark*, otherwise the salts will be precipitated. Then treat with strong alcohol for twelve to twenty-four hours, and make sections.

The rationale of this mixture is, that it fixes tissues faithfully, without causing the production of the delusive reticular precipitates of albuminoids which we have mentioned as being produced by chromic acid—that is the part played by the bichromate and sulphate; and that it also fixes faithfully the chromatin of nuclei—that is the part played by the organic acid.

The Chromates are useful as hardening rather than fixing agents. They have a very mild and even action on tissues, but are not at all penetrating and act very slowly. They may still be found useful for fixing certain tissues, some of those of Mollusca, for example. For mixtures that may be used for such a purpose, *see* the chapter on HARDENING AGENTS.

44. Cupric Sulphate.—Cupric sulphate was recommended some few years ago in one of the well-known handbooks. It was recommended (in a place which I cannot now find) to be used for marine organisms in saturated solution in sea-water, the organisms to be preserved in the solution itself till wanted. It has been quite recently (*Arch. d. Sci. phys. et nat.*, Juin, 1889, t. xxi, p. 556) recommended by BEDOT for the preparation of Siphonophora and other delicate pelagic animals. *See* "*Siphonophora.*"

FRIEDLAENDER (*Biol. Centralbl.*, x, 1890, p. 483; *Journ. Roy. Mic. Soc.*, 1890, p. 804) preserves this class of objects by inundating them with a mixture of 125 parts cupric sulphate, 125 parts zinc sulphate, and 1000 parts water.

45. Alum.—Alum has been used for fixing purposes, and may therefore be mentioned here. Although quite superseded for general work by other reagents, it may possibly still be found useful for certain special purposes. For instance, for the preservation of *Medusæ* the following process has been recommended (by Pagenstecher). Take two parts of common salt and one of alum, and make a strong solution. Throw the animals into it alive, and leave them there for twenty-four to forty-eight hours. Preserve in weak alcohol. A saturated solution of alum in sea-water preserves very well the forms of *Salpidæ*, *Medusæ*, *Ctenophora*, and other pelagic animals. It constitutes a preservative medium in which the objects may remain till wanted.

According to my experience, it is not to be recommended for any but the very coarsest work. It should be noted, however, that Ranvier (*Traité Technique*, p. 279) found that it fixed cartilage-cells better than any other reagent. He employed a solution of 0·5 per cent.

46. Permanganate of Potash (DU PLESSIS, *Bull. Soc. Vaud. Sci. Nat.*, 2, sér. xv, pp. 278—280, 1878).—A strong solution in water. I find this reagent has very slight penetrating power, and, besides, macerates some tissues. It is therefore not adapted for general use, but it preserves very well the forms of cells, and has one great virtue—it kills, I fancy, more rapidly than any other agent I have been able to find; even 2 per cent. osmic acid is not equal to it in this respect. I have found it sometimes very valuable for the study of isolated and very contractile cells, such as some spermatozoa.

CHAPTER V.

FIXING AGENTS—CHLORIDES, ORGANIC ACIDS, AND OTHERS.

Chlorides.

47. Bichloride of Mercury (Corrosive Sublimate).—Corrosive sublimate is stated in the books to be soluble in about sixteen parts of cold and three of boiling water. It will probably be found that the aqueous solution contains about 5 per cent. of the sublimate at the temperature of the laboratory. It is more soluble in alcohol than in water, and still more so in ether. Its solubility in all these menstrua is augmented by the addition of hydrochloric acid, ammonious chloride, or camphor. With sodium chloride it forms a more easily soluble double salt; hence sea-water may dissolve as much as 15 per cent., and hence the composition of the liquid of Lang.

For fixing corrosive sublimate may be, and very frequently is, used pure; but in most cases a finer fixation will be obtained if it be acidified with acetic acid, say about 1 per cent. of the acid. I find that a *saturated solution in 5 per cent. acetic acid* is a very good formula for marine animals. Van Beneden has recommended a saturated solution in 25 per cent. acetic acid.

In either case, the most concentrated solution obtainable should in general be taken. The cold saturated aqueous solution will suffice in most cases; but for some very contractile forms (coral polypes, Planaria), a concentrated solution in warm or even boiling water should be employed. For Arthropoda the alcoholic solution is frequently indicated. Delicate objects, however, may require treatment with weak solutions. Harting found solutions of 0·2 to 0·5 per cent. suitable for blood-corpuscles, and Pacini's fluids are much of the same strength. For these see the chapter on Examination Media.

Objects should in all cases be removed from the fixing bath

as soon as fixed, that is, in other words, as soon as they are seen to have become opaque throughout, which is practically as soon as they are penetrated by the liquid. Small objects are fixed in a few minutes. I have found that a "salivary" gland of the larva of *Chironomus* is thoroughly fixed in three seconds.

Wash out with water or with alcohol. I consider alcohol almost always preferable. Alcohol of about 70 per cent. may be taken. The extraction of the sublimate is hastened by the addition of a little camphor to the alcohol. Or a little tincture of iodine may be added to the liquid, either alcohol or water, used for washing, and the liquid changed until it no longer becomes discoloured by the objects. It is important that the sublimate be thoroughly removed from the tissues, otherwise they become brittle. They will also become brittle if they are kept long in alcohol.

You may stain in any way you like. Carmine stains are peculiarly brilliant after sublimate, owing to the formation of mercuric carminate. It is not necessary that the objects be thoroughly washed out before staining; the staining processes themselves may be made to constitute a part of the washing-out process.

It must be remembered that the solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass, or platinum may be used; for dissecting them, hedgehog spines, or quill pens.

When properly employed, sublimate is undoubtedly a fixing agent of the very highest order. It is applicable to most classes of objects. It is perhaps less applicable, in the pure form, to Arthropods, as it possesses no great power of penetrating chitin.

Before passing to the sublimate mixtures that have been recommended, it must here be stated that *for general purposes* there seems to be no use in adding anything except a little acetic acid to the pure sublimate.

48. Corrosive Sublimate (LANG's formula, '*Zool. Anzeiger*,' 1878, i, p. 14). For *Planaria*.—Take—

Distilled water . . .	100 parts by weight.
Chloride of sodium . .	6 to 10 parts.
Acetic acid	6 to 8 „
Bichloride of mercury . .	3 to 12 „
(Alum, in some cases . .	½).

The Planaria are to be placed on their backs and the mixture is to be poured over them. They die extended. After the lapse of half an hour they are brought into alcohol, first of 70 per cent., then of 90 per cent., then absolute, and in two days' time are sufficiently hardened.

Second formula (*ibid.*, 1879, ii. p. 46).—Make a concentrated solution of corrosive sublimate in picro-sulphuric acid, to which has been added 5 per cent. of acetic acid.

A solution containing 5 g. sublimate, 0·5 g. sodium chloride, and 100 c.c. water, has been quoted as "solution of GAULE."

49. Cyanide of Mercury (KEISER, *Biblioth. Zool.*, H. vii, 1 Hälfte, 1891; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 363).—Saturated aqueous solution. Keiser found that this solution, warmed to 45° to 50° C., and allowed to act for from 15 to 60 minutes, and then washed out with 70 per cent. alcohol, was the best of all fixing media for *Acanthocephali*.

50. Chloride of Platinum (Platinic Chloride, $PtCl_4$).—An *extremely valuable* reagent, originally introduced for the study of karyokinesis, but of general application. RABL, to whom we owe the introduction of this agent, employs an aqueous solution of 1·300. The objects remain in it for twenty-four hours, and are then washed with water, hardened in alcohol, and sectioned. Stain with Delafield's hæmatoxylin, or with safranin.

The action of platinum chloride is similar to that of gold chloride, with the advantage that there is no blackening of the preparations. Rabl finds it give better results (for the study of karyokinesis) than any other reagent except chromoformic acid (§ 32). It causes a slight shrinkage of the chromatin elements, a condition that renders the granules of Pfitzner and the longitudinal division of the elements very distinctly visible (see Rabl's well-known paper in *Morph. Jahrb.*, Bd. x, 1884, p. 216).

Platinum chloride is an extremely deliquescent salt, and for this reason had better be procured in solution. Ten per cent. solutions are found in commerce.

For *Merkel's* solution (chromo-platinic mixture) *see ante*, § 42.

51. Platino-Aceto-Osmic Mixture (HERMANN, *Arch. f. mik. Anat.*, xxxiv, 1889, p. 58).—The author obtained excellent results by substitutng 1 per cent. platinic chloride for the chromic acid in Flemming's *strong* formula for chromo-aceto-osmic acid (§ 36), the other ingredients either remaining as

before, or the osmium being diminished one half. Thus, 1 per cent. platinic chloride 15 parts, glacial acetic acid 1 part, and 2 per cent. osmic acid either 4 parts or only 2 parts. Hermann found that protoplasmic structures are thus better preserved than with the chromic mixture, which I think is undoubtedly the case. It was noted above (§ 30) that a chief objection to the use of chromic acid is that it precipitates certain of the liquid albuminoids of tissues in the form of filaments or networks, which are often of great regularity, and simulate structural elements of the tissues. *This platinum chloride does not do.* On the whole, I take it that this mixture is *probably the finest fixative yet discovered.* Unfortunately it is also the most expensive.

The after-treatment and staining should be the same as for objects treated with Flemming's solution.

The remarks in § 36, as to the deterioration of Flemming's solution by evaporation of osmium apply with equal force to Hermann's mixture.

52. Chlorides of Palladium, of Gold, and of Iron.

Palladium Chloride.—Palladium chloride has been recommended by experienced workers. It is used in solutions of 1·300, 1·600, or 1·800 strength, for from one to two minutes. Cattaneo recommends it as being the best of fixatives for Infusoria. Tissues are impregnated and coloured brown by it. For small objects one or two minutes will suffice for fixation.

This salt is found in commerce in the solid state. To dissolve it, take 10 grammes of the salt, one litre of water, and four to six drops of hydrochloric acid. Solution will be effected in twenty-four hours.

Gold Chloride.—When used for fixing (and not for the object of staining by impregnation) gold chloride is generally used in solution of ½ per cent. strength, for a few minutes (30 at most). Weaker solutions (⅕th per cent.) or stronger (1 to 2 per cent.) may also be used. Wash out with water.

Gold chloride is one of the most faithful fixing agents we know of. But it is not fitted for general work on account of the capricious fashion in which it undergoes reduction in the tissues, rendering the impregnated elements unsusceptible of staining.

Perchloride of Iron (Fol, *Zeit. f. wiss. Zool.*, Bd. xxxviii, 1883, p. 491; and *Lehrb. d. vergl. mik. Anat.*, p. 102).—Fol recommends 1 vol. of *Tinct. Ferri Perchlor.* P. B. diluted with 5 to 10 vols. of 70 per cent. alcohol. This gives better results than the weaker (2 per cent.) mixture at first recommended. Aqueous solutions do not give nearly so good results.

Fix for a short time only and wash with alcohol. The preparations are best stained with pyrogallol (see the chapter on Impregnation Methods). Fol recommends this process chiefly for Infusoria, and other ciliated objects, but

also as a general zoological method. I hear from Naples that it has been tried for the preservation of marine organisms, and found wanting. Its chief value seem to lie in the pyrogallol staining method, which may be found very useful for nerve-end organs, which are impregnated somewhat selectively by it.

Perchloride of iron (the tincture diluted with 3 to 4 vols. of either alcohol or water) has lately been recommended for fixing medullated nerve by Platner (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 187).

Organic Acids.

53. Acetic Acid.—The place of honour amongst organic acids considered as fixing agents appears rightfully to belong to this old-fashioned reagent. In the first edition of this work it was merely stated that acetic and formic acid "are useful and well-known fixatives of nuclei. Flemming,, who has made a special investigation of their action, finds (*Zellsubstanz*, &c., p. 380) that the best strength is from 0·2 to 1 per cent. Strengths of 5 per cent. and more bring out the nuclein structures clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths" (*ibid.*, p. 103). It must now be stated that, thanks to v. Beneden, the *strong* acid has become established as a most precious fixative of the most varied zoological objects. It is particularly applicable to very contractile objects, such as many Vermes, Cœlenterata, and Nudibranchs; it kills with the utmost rapidity, *and has a tendency to leave them fixed in the state of extension.* The *modus operandi* is in general as follows:—Pour glacial acetic acid in liberal quantity over the organisms, leave them until they are penetrated by it—which should be in five or six minutes, as the strong acid is a highly penetrating reagent—and wash out in frequent changes of alcohol of gradually increasing strength. Some persons begin with 30 per cent. alcohol, but this appears to me rather weak, and I think 70 per cent. or at least 50 per cent. alcohol should be preferred.

In the *Traité des Méth. Techn.*, 1887, I stated that the reason why glacial acetic acid was not more used was that it did not faithfully preserve delicate histological and cytological detail. I now believe that if the instructions above given be followed, in particular as regards the employment of the *glacial* acid, and the washing out with somewhat strong alcohol, the most

delicate detail will generally be found admirably preserved. I see no reason why other energetic reagents should not be combined with the glacial acetic acid if desired. Dr. LINDSAY JOHNSON (*in. litt.*) has found that the best of all fixatives for retina is a mixture of equal parts glacial acetic acid and 2 per cent. osmic acid.

54. Acetic Alcohol (CARNOY, *La Cellule*, t. iii, 1, 1886, p. 6; and *ibid.*, 1887, 2, p. 276; v. BENEDEN et NEYT, *Bull. Ac. roy. d. sci. de Belg.*, t. xiv, 1887, p. 218; ZACHARIAS, *Anat. Anz.*, iii Jahrg., 1, 1888, pp. 24—27; v. GEHUCHTEN, *ibid.*, 8, p. 237). —CARNOY has given two formulæ for this important reagent. The first is—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	3 parts.

The second is—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	6 parts.
Chloroform	3 „

The addition of chloroform is said to render the action of the mixture more rapid.

V. BENEDEN and NEYT take equal volumes of glacial acid and absolute alcohol.

ZACHARIAS takes—

Glacial acetic acid . . .	1 part.
Absolute alcohol . . .	4 parts.
Osmic acid	A few drops.

Acetic alcohol is one of the most penetrating and quickly acting fixatives known. It preserves nuclei admirably, and admits of admirable staining in any way that may be preferred. It was imagined by all of the authors quoted for the study of karyokinesis in the ova of *Ascaris*,—proverbially one of the most difficult objects to fix,—but it is applicable to tissues in general. You may wash them out with alcohol and treat them afterwards in any way that may be preferred. It will be well, however, to avoid treatment with water as much as possible.

55. Formic Acid may be used dilute in the same way as acetic acid (*supra*, § 53). It is probable that it might also take the place of acetic acid in the concentrated form, but I am not aware of any experiments in this direction.

56. Picric Acid.—Picric acid should always be employed in the form of a *strong* solution. (That is to say, strong solutions must always be employed when it is desired to make sections or other preparations of tissues with the elements *in situ*, as weak solutions macerate; but for dissociation preparations or the fixation of isolated cells, weak solutions may be taken. Flemming finds that the fixation of nuclear figures is equally good with strong or weak solutions.) The saturated solution is the one most employed. (One part of picric acid dissolves in about 75 parts of cold water; in hot water it is very much more soluble.) Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria one to at most two minutes will suffice, whilst objects of a thickness of several millimètres require from three to six hours' immersion.

Picric acid should *always be washed out with alcohol*, as water is hurtful to tissues that have been prepared in it. For the same reason, during all remaining stages of treatment, water should be avoided; staining should be performed by means of alcoholic solutions, the only exceptions to this rule being in favour of picro-carmine, which, probably on account of the picric acid contained in it, does not appear to exert so injurious an influence as other aqueous stains, and of methyl green, and some few other aqueous stains that are themselves weak hardening agents. It is one of the advantages of picric acid that, by sufficiently prolonged soaking, it can with certainty be entirely removed from any tissue by means of alcohol.

Tissues fixed in picric acid can, after removal of the acid by soaking, be perfectly stained in any stain. Mayer's paracarmine, Grenacher's alcoholic borax-carmine, or Mayer's hæmacalcium may be recommended.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures. For such objects alcohol of 70 per cent. to 90 per cent. should be taken for washing out, and staining should be done by means of Mayer's cochineal or hæmacalcium.

In very many if not most cases it is advantageous to employ picric acid in the manner suggested by Kleinenberg (*see* below), that is, in combination

with sulphuric acid ; or with nitric acid, or hydrochloric acid, as suggested by P. Mayer (*see* below).

57. Picro-sulphuric Acid (KLEINENBERG, *Quart. Journ. Mic. Sci.*, April, 1879, p. 208; MAYER, *Journ. Roy. Mic. Soc.* (N.S.), ii (1882), p. 867).—By picro-sulphuric acid, without any qualifying term, I understand a fluid made (following Mayer, l. c.) as follows :—Distilled water, 100 vols. ; sulphuric acid, 2 vols. ; picric acid, as much as will dissolve (this will be about 0·25 vol. ; as the picric acid is much less soluble in sulphuric acid solution than in water). This may also, in any case in which confusion is likely to arise, be called "concentrated" or "undiluted picro-sulphuric acid."

By "liquid of Kleinenberg" I understand a mixture suggested by Kleinenberg (l. c.), and best made by diluting the concentrated picro-sulphuric acid prepared as above with three times its volume of water.

(Kleinenberg also directed the addition of as much creosote as would mix. This was done with the idea of eliminating the swellings produced in some objects by the liquid, but it has been found not to have the effect attributed to it, and has been abandoned. FOL (*Lehrb.*, p. 100) states that the same end may be attained by adding one third vol. of 1 per cent. chromic acid.)

Of these two formulæ the one commonly employed is that given by Kleinenberg,—the dilute mixture ; undiluted picro-sulphuric acid being reserved for objects requiring special treatment, chiefly Anthropods. I may as well say at once that in my opinion this practice is erroneous, for I hold that Kleinenberg's solution is much weaker than is desirable in the majority of cases, and should be reserved for special cases, such perhaps as that for which it was originally proposed, the embryology of the earthworm ; and the concentrated solution should be the one taken for general work. *This particularly applies to marine organisms.*

The treatment is the same in either case. "The object to be preserved should remain in the liquid for three, four, or more hours ; then it should be transferred, in order to harden it and remove the acid, into 70 per cent. alcohol, where it is to remain five or six hours. From this it is to be removed into 90 per cent. alcohol, which is to be changed until the yellow tint has either disappeared or greatly diminished."

Warm alcohol extracts the acid much more quickly than

cold, with which *weeks* may be required to fully remove the acid from chitinous structures. I call attention here to what was said as to washing out under the head of *picric acid*, viz. that washing out must *never be done with water*. This is a most important point, and one that is not sufficiently attended to. You may stain as directed above for picric acid. You may, of course, stain sections with alcoholic solutions of safranin or the like.

The advantages of picro-sulphuric acid as a fixing agent are, that it kills tissues very rapidly, that it has great penetrating power, that it can be totally soaked out of the structures with alcohol (it is much more easily removed from the tissues than pure picric acid), leaving them in a good condition for staining, and, in the case of marine organisms, that it effectually removes the different salts of sea-water that are present in them.

It has many disadvantages. For vertebrata it should be used with caution, on account of the swelling caused by sulphuric acid in connective tissue. For structures that contain much lime it is not to be recommended, for it dissolves the lime and throws it down as crystals of gypsum in the tissues. (For such structures the picro-nitric or picro-hydrochloric acid is to be preferred.) In numberless cases it produces swellings and maceration. For the preservation of delicate, watery organisms, such as Medusæ, it is an abomination. For cytological researches it should be avoided, as its action on both cytoplasm and nuclei is frequently most injurious. On the whole, I find that for such objects as Arthropoda it is valuable on account of its great penetrating power, the possibility of removing the acid entirely by washing, and the facility thereby given for staining *in toto*. But *for general work, I consider that it is one of the most overrated reagents that ever came into favour through the prestige of authority.*

58. Picro-nitric Acid (Mayer, *Mitth. Zool. Stat. Neapel*, 1881, p. 5; *Journ. Roy. Mik. Soc.* (N.S.), ii, 1882, p. 868).—Prepared in the same way as picro-sulphuric acid except that instead of 2 vols. sulphuric acid you take 5 vols. pure nitric acid (of 25 per cent. N_2O_5). Mayer now dissolves the picric acid in the nitric acid water, so that the formula runs:

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5) . . .	5 „
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are very similar to those of picro-sulphuric acid, with the advantage of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. "Mayer recommends it strongly, and states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric, or picro-sulphuric acid."

59. Picro-hydrochloric Acid (Mayer, *ibid.*).—Prepared in the same way as picro-sulphuric acid, except that instead of 2 vols. of sulphuric acid

you take "8 vols. of pure hydrochloric acid of 25 per cent. HCl." Mayer now dissolves the picric acid in the hydrochloric acid water, so that the formula runs:

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl) . .	8 „
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are similar to those of picro-nitric acid.

59a. Picro-chromic Acid. *See ante*, § 41.

60. Picro-chromo-sulphuric Acid (Keiser, *Biblioth. Zool.*, H. vii, 1 Hälfte, 1891; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 363).—Keiser found the following mixture excellent for Acanthocephali:

Picric acid	1 gramme.
Conc. sulphuric acid	10 grammes.
Chromic acid	1 gramme.
Water	1000 grammes.

To be warmed to 55° C., allowed to act for 15 to 20 minutes, washed out for 5 to 10 minutes with hot water, and afterwards for some days in 60 per cent. alcohol.

61. Picro-osmic Acid.—Flemming (*Zells. Kern-u.-Zellth.*, p. 381) has experimented with mixtures made by substituting picric for chromic acid in the chromo-osmic mixtures (*ante*, §§ 34 and 35). The results are identical so far as regards the fixation (of nuclei); but staining is rendered more difficult.

62. Picric Alcohol (Gage, *Proc. Amer. Soc. Micr.*, 1890, p. 120; *Journ. Roy. Mic. Soc.*, 1891, p. 418).—Alcohol (95 per cent.), 250 parts; water, 250 parts; picric acid, 1 part. Fix for about 24 hours, wash out for a day in alcohol of 67 to 70 per cent., and then for a day or longer in alcohol of 75 to 82 per cent.

Other Fixing Agents.

63. Alcohol.—For fixing, only two grades of alcohol are found generally useful—very weak alcohol on the one hand, and absolute alcohol on the other hand. Absolute alcohol ranks as a fixing agent because it kills and hardens with such rapidity that structures have not time to get deformed in the process by the energetic dehydration that unavoidably takes place. Dilute alcohol ranks as a fixing agent in virtue of being of such a strength as to possess a sufficiently energetic coagulating action and yet contain enough water to have but a feeble and innocuous dehydrating action. The intermediate grades do not realise these conditions, and therefore should not be employed alone for fixing. But they may be very useful in combination with other fixing agents (such as cor-

rosive sublimate, chromic acid or nitric acid) by greatly enhancing their penetrating power; 70 per cent. is a good grade for this purpose.

One-third Alcohol.—The one grade of weak alcohol that is found generally useful for fixing is one-third alcohol, or RANVIER'S ALCOHOL, known in France as "Alcohol au tiers," which is the name given to it by Ranvier himself; in Germany as "Drittelalcohol" or "Ranviersche alcohol dilutus;" in Italy, as "alcool al terzo." It consists of *two parts of water and one part of alcohol of* 90 *per cent.* (and *not* of absolute alcohol, as was stated by an oversight in the first edition—an error which I have seen copied in more than one place). *See* the *Traité Technique* of Ranvier, p. 241, *et passim.*

Care should be taken that the alcohol is of the strength specified, as the effects of this reagent depend to a remarkable degree on its strength.

Objects may be left for twenty-four hours in this alcohol; not more, unless there be no reason for avoiding maceration, which will generally occur after that time. You may conveniently stain with picro-carmine, alum-carmine, or methyl green.

This classical reagent is a very mild fixative. Its hardening action is so slight that it is seldom indicated for the fixing of objects that are intended to be sectioned. Its chief use is for extemporaneous and dissociation preparations.

Absolute Alcohol.—This is also a very valuable reagent It preserves very well the structure of nuclei, which is by no means the case with one-third alcohol. It has over the latter also the advantage of superior penetrating power, being indeed one of the most penetrating of known fixing agents. Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration brought about by the slowness of penetration of common cold alcohol (especially in the case of Tracheata).

It is important to employ for fixing a very large proportion of alcohol. Alum-carmine is a good stain for small specimens so fixed. For preservation, the object should be put into a weaker alcohol, 90 per cent. or less.

Absolute alcohol is found in commerce. It is a product that it is almost impossible to preserve in use, on account of the rapidity with which it

hydrates on exposure to air. Fol recommends that a little quicklime be kept in it. This absorbs part at least of the moisture drawn by the alcohol from the air, and has the further advantage of neutralising the acid that is frequently present in commercial alcohol.

Another plan that I have seen recommended is to suspend strips of gelatin in it. It is stated that by this means ordinary alcohol may be rendered absolute.

Ranvier adopts the following plan for preparing an alcohol absolute enough for all practical purposes. Strong (95 per cent.) alcohol is treated with calcined cupric sulphate, with which it is shaken up and allowed to remain for a day or two. It is then decanted and treated with fresh cupric sulphate, and the operation is repeated until the fresh cupric sulphate no longer becomes conspicuously blue on contact with the alcohol; or until, on a drop of the alcohol being mixed with a drop of turpentine, no particles of water can be seen in it under the microscope. The cupric sulphate is prepared by calcining common blue vitriol in a porcelain capsule over a spirit lamp or gas burner until it becomes white, and then reducing it to powder (*see Proc. Acad. Nat. Sci. Philad.*, 1884, p. 27; *Science Record*, ii, 1884, p. 65; *Journ. Roy. Micr. Soc.* (N.S.), iv, 1884, pp. 322 and 984).

Acidulated Alcohol (Paul Mayer, *Mitth. Zool. Stat. Neapel*, ii, 1881, p. 7).—To 97 vols. of 90 per cent. alcohol, in which is dissolved a small quantity of picric acid, add 3 vols. pure hydrochloric acid. Leave the specimens in the mixture only just long enough to ensure that they are thoroughly penetrated by it. Wash out with 90 per cent. alcohol, the disappearance of the yellow stain of the picric acid being a sign that all the acid is removed.

The use of this mixture is for the preparation of coarse objects it is intended to preserve in alcohol. The object of the acid is to prevent both that glueing together of organs by the perivisceral liquid, which is often brought about by the coagulating action of pure alcohol, and the precipitation on the surface of organs of the salts contained in sea-water, which is a hindrance not only to the penetration of the alcohol, but also to subsequent staining.

Whitman (*Journ. Roy. Mic. Soc.* (N.S.), ii, 1882, p. 870) states that "acid alcohol as above prepared loses its original qualities after standing some time, as ether compounds are gradually formed at the expense of the acid." He also states that 70 per cent. alcohol may be taken instead of 90 per cent. for washing out.

Acetic Alcohol. See *ante*, § 54, and the chapter on Cytological Methods in Part II.

64. Chloride and Acetate of Copper (*Ripart et Petit's formula*, Carnoy, *La Biologie Cellulaire*, p. 94).

Camphor water (not saturated) . . .	75 grammes.
Distilled water	75 „
Crystallised acetic acid	1 gramme.
Acetate of copper	0·30 „
Chloride of copper	0·30 „

This is a very moderate and delicate fixative. I consider that it has not sufficient hardening power for objects that are intended to be dehydrated

and mounted in balsam, but is frequently excellent and sometimes indispensable for objects that are to be studied in as fresh a state as possible in aqueous media. Objects fixed in it stain instantaneously and perfectly with methyl green. Osmic acid may be added to the liquid to increase the fixing action. *For cytological researches* this is a most invaluable medium.

65. Acetate of Uranium (SCHENK, *Mitth. a. d. Embryol. Inst. Wien*, 1882, p. 95; cf. GILSON, *La Cellule*, 1, 1885, p. 141).—This reagent is very similar in its properties to picric acid. It has a mild fixing action, and a high degree of penetration, which may make it useful for Arthropoda. It may be combined with methyl green, which it does not precipitate.

66. Iodine.—Iodine possesses considerable hardening properties, and a very high degree of penetration; and, in point of fact, iodised serum, which is generally employed as an "indifferent liquid," that is, one which is supposed to exert no action whatever on tissues, is, in reality, a feeble hardening agent, and forms a most admirable fixing agent for delicate tissues. It is so classed by Ranvier (*see* **Iodised Serum**, Chap. XIX). KENT (*Manual of the Infusoria*, 1881, p. 114; *Journ. Roy. Mic. Soc.* (N.S.), iii, 1883, p. 730), has found it to act in a manner almost identical with osmic acid, and in some instances even more efficiently (for fixing Infusoria). His instructions are as follows:—"Prepare a saturated solution of potassic iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria."

Or you may use the solution of LUGOL, of which the formula is as follows:

Water	100 parts.
Iodide of potassium	6 „
Iodine	4 „

Iodine certainly kills cells very rapidly, without deforming them. Personally I have found it very useful for the examination of spermatozoa. Unfortunately I am not acquainted with any nuclear stain that will work well with it.

Very small objects may be instantaneously fixed by means of **vapour of iodine**. Crystals of iodine may be heated in a test-tube till the vapours are given off; then on inclining the tube the heavy vapours may be made to flow over the objects arranged on a slide. The slide should then be warmed to about 40° C. for 2 or 3 minutes in order to evaporate the iodine from the objects, which may then be mounted or otherwise treated as desired (OVERTON, *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 14).

CHAPTER VI.

HARDENING REAGENTS.

67. The Obligation of Hardening.—Methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is now, in the majority of cases, no longer necessary; by careful infiltration with paraffin or some other good infiltration mass, most soft objects can be satisfactorily cut with no greater an amount of previous hardening than is furnished by the usual passing of the tissues after fixing through successive alcohols in order to prepare them for the paraffin bath. But there are some exceptions. Such are, for instance, the cases in which it is desired to cut very large sections, such as sections of the entire human brain. Such an organ as this cannot be duly infiltrated with alcohol in a few hours, and it is doubtful whether it can be duly infiltrated with paraffin or any other imbedding mass in any reasonable time. And certain organs that are either extremely delicate or inaccessible, such as retina or cochlea, will require to be specially hardened in order to give the best results. The processes employed for hardening such specimens as these will be described when treating of the organs in question. In this chapter, which may be considered as parenthetical, I confine myself to such general statements concerning the employment of the usual hardening agents as appear likely to be generally useful.

68. The Practice of Hardening—Hints and Cautions.—Employ in general a relatively large volume of hardening liquid, and change it very frequently. The exact proportions may be made out by experiment for each reagent and each class of objects. If the volume of liquid be insufficient its composition will soon become seriously altered by the diffusion into it of the soluble substances of the tissues; and the result may be

a macerating instead of a hardening liquid. Further, as soon as, in consequence of this diffusion, the liquid has acquired a composition similar in respect of the proportions of colloids and crystalloids contained in it to that of the liquids of the tissues, osmotic equilibrium will become established, and diffusion will cease. That is to say, the hardening liquid will cease to penetrate. This means, of course, maceration of internal parts. On the other hand, it appears that a certain slight proportion of colloids in the hardening liquid is favorable to the desired reaction, as it gives a better consistency to the tissues by preventing them from becoming brittle. Hence the utility of employing *a certain proportion* of hardening agent.

Hardening had better be done in tall cylindrical vessels, the objects being suspended by a thread at the top of the liquid. This has the advantage of allowing diffusion to take place as freely as possible, whilst any precipitates that may form fall harmlessly to the bottom.

Always begin hardening with a weak reagent, increasing the strength gradually, as fast as the tissues acquire a consistence that enables them to support a more energetic action of the reagent.

Let the objects be removed from the hardening fluid as soon as they have acquired the desired consistency.

As to the choice of a hardening reagent, if you wish, above all, for a rapid and energetic action, take chromic acid. If you wish for a more moderate and more equable action, take a chromic salt, or one of the compounds of which the chromic salts are the principal ingredients.

Mineral Acids.

69. Chromic Acid.—Chromic acid is generally employed in strengths of $\frac{1}{5}$th per cent. to $\frac{1}{2}$ per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days; brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grammes for a piece of tissue of 1 centimètre cube, Ranvier).

In order to obtain the best results you should not employ

portions of tissue of more than an inch cube. For a human spinal cord you should take two litres of solution, and change it for fresh after a few days. Six weeks or two months are necessary to complete the hardening.

The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. (These precautions are peculiarly necessary in the case of chromic acid.) They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. I think it is frequently useful to add a little glycerin to the hardening solution, there is less brittleness and, I think, less shrinkage.

The reader's attention is called to the statements made in § 30 concerning the action of light on the alcohol containing chromic objects.

Further directions for the employment of chromic acid will be given in the special paragraphs. Chromic acid is a most powerful and rapid hardening agent (by it, you may obtain in a few days a degree of hardening that you would hardly obtain in as many weeks with bichromate, for instance.) It has the defect of a great tendency to cause brittleness.

70. Chromic Acid and Spirit (URBAN PRITCHARD, *Quart. Journ. Mic. Sci.*, 1873, p. 427).—Chromic acid, 1 part; water, 20 parts; rectified spirit, 180 parts. Dissolve the chromic acid in the water first, and then add the spirit (violent action will ensue if the dry chromic acid be added directly to the spirit). The colour of the solution soon becomes brown. If, after a few days, it turns semi-gelatinous, it should be changed for fresh. From a week to ten days is required to harden such tissues as retina, cochlea, &c., for which this fluid is particularly well adapted.

71. Chromo-osmic Acid (MAX FLESCH.) **Chromo-aceto-osmic Acid** (FLEMMING.)—Either of these mixtures may be used for prolonged hardening, and are admirable. The weak form of Flemming's solution is the one that should generally be taken for hardening purposes. (*See* §§ 34 and 35.)

For delicate objects perhaps even better results may be obtained by means of **Chromic Acid and Platinic Chloride** (MERKEL'S Solution). *See* § 42, *ante*.

72. Picro-chromic Acid.—This fixative may be found useful for

hardening objects that are only penetrable with difficulty. Some Tunicata, for instance. *See ante*, § 41.

73. Osmic Acid.—Osmic acid is much more useful as a fixing agent than as a hardening agent. Long immersion in osmic acid is sure to cause blackening, and may cause brittleness in the tissues. The strengths employed for hardening vary from $\frac{1}{5}$th per cent. to 1 per cent., and the tissues are left in the solutions for twelve to twenty-four hours, seldom more. The chief use of osmium in hardening is to enhance the energy of action of certain mixtures into which it enters as a component, and serves to materially shorten the time necessary for hardening. See the further information as to the employment of this reagent given above, §§ 25, 26, 27, 28, 29.

74. Nitric Acid.—Nitric acid is taken of a strength of from 3 per cent. to 10 per cent. or more, and may be allowed to act for two or three weeks. It gives, thus employed (10 per cent. to 12 per cent.), very tough preparations of brain. It is also conveniently used by employing a very short immersion and completing the hardening with alcohol, in which case it is properly considering as a fixing agent. See the information given under this head, § 37, *ante*.

Salts.

75. Bichromate of Potash.—Perhaps the most important of all known hardening agents, *sensu stricto*. It hardens slowly, much more so than chromic acid, but it gives an incomparably better consistency to the tissues, and it has not the same tendency to make them brittle if the reaction be prolonged. They may remain almost indefinitely exposed to its action without much hurt.

The strength of the solutions employed is from 2 to 5 per cent. As with chromic acid it is extremely important to begin with weak solutions and proceed gradually to stronger ones. About three weeks will be necessary for hardening a sheep's eye in solutions gradually raised from 2 to 4 per cent. Spinal cord requires from three to six weeks; a brain, at least as many months.

After hardening, the objects should be well soaked out in water before being put into alcohol. They had better be kept in the dark when in alcohol (*see* above, § 30). *If you wish to have a good stain with carmine, especially ammonia-carmine, which is admirable for portions of nervous system so hardened, you should not put the objects into alcohol at all, even for a second, until they have been stained.*

You may stain either with carmine or hæmatoxylin.

Bichromate objects have an ugly yellow colour which cannot be removed by soaking in water. It is said that it can be removed by washing for a

few minutes in a 1 per cent. solution of chloral hydrate. Gierke, however, says that this treatment is prejudicial to the preservation of the tissues.

76. Müller's Solution.—

Bichromate of potash . .	2—2½ parts.
Sulphate of soda . . .	1 part.
Water	100 parts.

The duration of the reaction is about the same as with the simple solution of chromic salts.

This fluid was very highly in vogue for many years, but seems lately to be much less used. I fancy that the superiority of this mixture over the simple bichromate solution is not illusory, and is due to the formation in it of a trace of free chromic acid. Fol says that for mammalian embryos, for which it has been recommended, it is worthless.

77. Erlicki's Solution (*Warschauer med. Zeit.*, xxii, Nos. 15 and 18).—

Bichromate of potash . . .	2·5 parts.
Sulphate of copper . . .	1·0 part.
Water	100·0 parts.

Here the addition of the cupric sulphate is intelligible. This salt is itself a hardening agent of some energy, and may well serve to reinforce the somewhat slow action of the bichromate. As a matter of fact, "Erlicki" hardens very much more rapidly than either simple bichromate or Müller's solution. A spinal cord may be hardened in it in four days at the temperature of an incubator, and in ten days at the normal temperature (Fol, *Lehrb. d. vergl. mik. Anat.*, p. 106). I believe it to be one of the best hardening agents known for voluminous objects. Human embryos of several months may be conveniently hardened in it.

Nerve-centres that have been hardened in Erlicki's fluid frequently contain dark spots with irregular prolongations, simulating ganglion-cells. These were at one time taken to be pathological formations, but they are now known to consist of precipitates formed by the action of the hardening fluid. They may be removed by washing with hot water, or with water slightly acidified with hydrochloric acid, or by treating the specimens with 0·5 per cent. chromic acid before putting them into alcohol (Tschisch, *Virchow's Arch.*, Bd. xcvii, p. 173; Edinger, *Zeit. f. wiss. Mik.*, ii, 2, p. 245; Loewenthal, *Rev. méd. de la Suisse romande*, 6me année, i, p. 20).

78. Bichromate and Platinic Mixture (LINDSAY JOHNSON'S Mixture; communicated by Dr. Lindsay Johnson).—

Bichromate of potash (2·5 per cent.) .	65 parts.
Osmic acid (2 per cent.) . . .	15 „
Platinic chloride (2 per cent.) . .	15 „
Acetic or formic acid	5 „

This mixture was imagined for the preliminary hardening of retina, being allowed to act for two hours only, and then being followed by final hardening in pure bichromate solution. But I have no doubt that it will prove applicable to other structures, and give excellent results as a slow and gentle hardening agent. For this purpose I would suggest that the osmium might be diminished or omitted, the proportion of Pt. Cl_4 increased, and that of acetic acid diminished. The function of the osmic acid in the present formula is to enhance the hardening energy of the mixture. Dr. Lindsay Johnson writes me that "it greatly reduces the length of time necessary for hardening, three days being the time from removal of the organ to its being in celloidin under dilute spirit."

"If the osmium has a tendency to blacken, this may be entirely prevented and a beautiful delicate chestnut-brown deepening towards Bartholozzi-red tint obtained by adding ten parts of 5 per cent. solution of nitrate of uranium, which forms a layer of uranium on the top of the reduced platinum and osmium (one or both)."

79. Bichromates and Alcohol.—Mixtures of either bichromate with alcohol may be employed, and have a more rapid action than the aqueous solution. Thus HAMILTON takes for hardening brain a mixture of 1 part methylated spirits with 3 parts of solution of Müller (see the chapter on the Central Nervous System in Part II.; see also KULTSCHIZKY'S Mixture, *ante*, § 43). Preparations should be kept in the dark during the process of hardening in these mixtures.

80. Bichromate of Ammonia.—A review of the literature of the subject shows that this salt is in considerable favour, for what precise motive is not apparent. Its action is very similar to that of the potassium salt. Fol says that it penetrates somewhat more rapidly, and hardens somewhat more slowly. It should be employed in somewhat stronger solutions, up to 5 per cent.

81. Neutral Chromate of Ammonia is preferred by some anatomists.

It is used in the same strength as the bichromate. Klein has recommended it for intestine, which it hardens, in 5 per cent. solution, in twenty-four hours.

82. Sulphate of Copper.—This salt is seldom used alone, perhaps because it does not give a sufficiently favorable consistency to the tissues hardened by it. I take from the *Lehrbuch* of Fol (p. 106) the following formula, which was first published by Remak, then modified by Goette, and is said to be useful for hardening the ova of Amphibia:

2 per cent. solution of sulphate of copper . .	50 c.c.
Alcohol of 25 per cent.	50 c.c.
Rectified wood vinegar	35 drops.

Chlorides and others.

83. Platinic Chloride Mixture (Merkel's Solution).—The formula of this admirable reagent has been given above, § 42. It is an admirable hardening medium for delicate objects. Merkel states that he allowed from three to four days for the action of the fluid for the retina; for Annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds "one hour sufficient, and transfers to 50 per cent. alcohol."

Whitman recommends, for the hardening of pelagic fish ova, a stronger mixture (due, I believe, to Eisig), viz.:

0·25 per cent. solution of platinum chloride .	1 vol.
1 per cent. solution of chromic acid . .	1 „

The ova to remain in it one or two days (Whitman, *Methods in Micro. Anat.*, p. 153).

84. Palladium Chloride (F. E. Schultze, *Arch. mik. Anat.*, iii, 1867, p. 477).—This reagent was recommended by Schultze partly as giving to tissues a better consistency than chromic acid or Müller's solution, and partly on account of a special faculty for penetrating organs rich in connective tissue that he attributes to it. It is an impregnation reagent, staining certain elements of tissues in various tones of brown. For the somewhat lengthy details of the manner of employing it, the reader is referred to the paper quoted.

85. Chloride of Zinc is only employed for brain, see *post*, Part II (Giacomini).

86. Picric Acid taken alone is a weak hardening agent, little used. It should be employed in saturated solution. But it is a useful ingredient in mixtures, serving to enhance the penetrating power. See **Picro-chromic Acid**, *ante*, § 72; also Gage's **Picric Alcohol**, § 62.

87. Acetate of Lead.—Both the neutral acetate (sugar of lead) and the basic acetate have been used for hardening nerve tissues. Anna Kotla-

REWSKY found that nerve-cells hardened in 10 per cent. solution of sugar of lead were admirably preserved. See her "Inaug.-Diss." in *Mitth. d. naturf. Ges. Bern.*, 1887, and *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 387.

88. Alcohol.—When used alone, alcohol is inferior as a hardening agent to most of the reagents discussed above; but when judiciously employed to complete the action of a good fixing agent, it renders most valuable services. 90 to 95 per cent. is the most generally useful strength. Weaker alcohol, down to 70 per cent., is often indicated. Absolute alcohol is seldom advisable. You ought to begin with weak, and proceed gradually to stronger, alcohol. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of the alcohol, in order to have the tissue constantly surrounded with pure spirit (the water and colloid matters extracted from the tissue falling to the bottom of the vessel). Many weeks may be necessary for hardening large specimens. Small pieces of permeable tissue, such as mucous membrane, may be sufficiently hardened in twenty-four hours.

89. Iodine may be used in combination with alcohol, and render service through its great penetrating power. See the method of BETZ, *post*, Part II.

90. Pyridin.—Pyridin has been lately recommended as a hardening agent (by A. DE SOUZA). It is said to harden, dehydrate, and clear tissues at the same time. They may be stained after hardening by anilin dyes dissolved in the pyridin, or passed through water and stained by the usual processes. It is said to harden quickly, and to give particularly good results with brain. See *Comptes Rendus hebd. de la Soc. de Biologie*, 8 sér., t. iv, No. 35, p. 622; *Zeit. f. wiss. Mik.*, v, i, 1888, p. 65; *Journ. Roy. Mic. Soc.*, 1888, p. 1054.

CHAPTER VII.

STAINING.

91. The Kinds of Stains.—Stains are either General or Special (otherwise called Specific, or Selective, or Elective). A general stain is one that takes effect on all the elements of a preparation. A special, specific, selective, or elective stain is one that takes effect only on some of them, certain elements being made prominent by being coloured, the rest either remaining colourless or being coloured with a different intensity or in a different tone. To obtain this *differentiation* is the chief object for which colouring reagents are employed in microscopic anatomy.

Two chief kinds of this selection may be distinguished,—histological selection, and cytological selection. In the former an entire tissue or group of tissue-elements is prominently stained, the elements of other sorts present in the preparation remaining colourless or being at all events differently stained, as in a successful impregnation of nerve-endings by means of gold chloride. This is the kind of stain that is generally meant by a *specific* stain. In the latter, the stain seizes on one of the constituent elements of cells in general, namely, either the nucleus or the extra-nuclear parts.

Stains that thus exhibit a selective affinity for the substance of nuclei, or *nuclear* stains, form at present by far the most important class of stains—in zootomy at any rate. What the zootomist wants, and the histologist too, in the great majority of cases, is either to differentiate the intimate structures of cells by means of a colour reaction, in order to study them for their own sakes, or to have the nuclei of tissues marked out by staining in the midst of the unstained material in such a way that they may form landmarks to

catch the eye, which is then able to follow out with ease the contours and relations of the elements to which the nuclei belong; the extra-nuclear parts of these elements being expressly left unstained in order that as little light as possible may be absorbed in passing through the preparation. Possibly this may be an irrational procedure, but it has hitherto been found in practice to be the most efficient for general work.

To these two chief kinds of selective stains may be added a third group, the *plasmatic* stains, consisting of those few stains that take effect on cytoplasm, or formed tissue, or ground substance *only*, leaving nuclei unstained. In this book, therefore, stains are looked upon as being (1) General or Ground stains; (2) Selective stains; the latter group being subdivided into (*a*) Nuclear, (*b*) Plasmatic, (*c*) Histologically Selective, or Specific. This classification, however, is not followed in the arrangement of the special paragraphs, it being more practical to follow an order based on the chemical nature of the staining agents, and on convenience of exposition.

Some writers have divided stains into nuclear, general, and selective. This arrangement appears to me faulty because every nuclear stain is *eo ipso* selective, and because it ignores the subdivisions of selective stains.

92. The Methods of Staining.—Colouring matters possessing so great an affinity for certain elements of tissues that they may be left to produce the desired electivity of stain, without any special manipulation on the part of the operator, are unfortunately rare. In practice, selective staining is arrived at in two ways. In the one, which may be called the *direct* method, you make use of a colouring reagent that stains the element desired to be selected more quickly than the elements you wish to have unstained; and you stop the process and fix the colour at the moment when the former are just sufficiently stained and the latter not affected to an injurious extent, or not affected at all, by the colour. This is what happens—for instance, when you stain the nuclei of a preparation by treatment with very dilute hæmatoxylin; you get, at a certain moment, a fairly pure nuclear stain; but if you were to prolong the treatment, the extra-nuclear elements would take up

the colour, and the selectivity of the stain would be lost. It may be noted of this method that it is in general the method of *fast* stains ("echte Farbung"), and that it renders great services in the colouring of specimens *in toto*,—a procedure which is not possible with the chief stains of the other class (the anilins). It is the old method of carmine and hæmatoxylin staining.

The second, or *indirect*, method, is the method of overstaining followed by partial discoloration. You begin by staining all the elements of your preparation indiscriminately, and you then wash out the colour from all the elements, except those which you desire to have stained, these retaining the colour more obstinately than the others in virtue of a certain not yet satisfactorily explained affinity. This is what happens —for instance, when you stain a section of one deep red in all its elements with safranin, and then treating it for a few seconds with alcohol, extract the colour from all but the chromatin and nucleoli of the nuclei. It is in this method that the coal-tar colours find their chief employment. It is in general applicable only to sections, and not to staining objects *in toto* (the case of borax-carmine is probably only a seeming exception to this statement). It is a method, however, of very wide applicability, and gives the most brilliant results that have hitherto been attained.

93. The State of the Tissues to be Stained.—It is generally found that precise stains can only be obtained with carefully fixed (*i. e.* hardened) tissues. Dead, but not artificially hardened tissues stain indeed, but not generally in a precise manner. Living tissue elements in general do not stain at all, but resist the action of colouring reagents till they are killed by them.

Staining "intra vitam."—Some few substances, however, possess the property of staining living cells without greatly impairing their vitality. Such are—in very dilute solutions —cyanin (or quinoleïn), methylen blue, Bismarck brown, anilin black, and, under certain conditions, dahlia, and gentian violet, with perhaps methyl violet and some others whose action is not yet sufficiently established by experiment. Congo, even in strong solution, is not toxic to some organisms, and stains some structures (see Scholtz, *Centralb. f. d. med. Wiss.*,

1886, p. 449; also *Journ. Roy. Mic. Soc.*, 1886, p. 1092). Living Rotifera are in part successfully stained by it during life. (The paper of Martinotti, *Zeit. f. wiss. Mik.*, v. 3, 1888, p. 305, may be consulted on this subject.)

As to the employment of these reagents, it may be noted that they must be taken in a state of extreme dilution, and in neutral or feebly alkaline solution—acids being of course toxic to cells. Thus employed, they will be found to tinge with colour the cytoplasm of certain cells during life (never, so far as I know, nuclear chromatin during life;—if this stain, it is a sign that death has set in). The stain is sometimes diffused throughout the general substance of the cytoplasm, sometimes limited to certain granules in it (which have been taken, perhaps without sufficient reason, to be identical with the granules of Altmann (Altmann's *Studien über die Zelle*, 1886). Methylen blue has the valuable point that it is perfectly soluble in saline solutions, and may therefore be employed with marine organisms by simply adding it to sea-water. The others are not thus soluble to a practical extent, but I find that gentian and dahlia become so if a trace of chloral hydrate —0·25 per cent. is amply enough—be added to the saline solution. Any of these reagents may be rubbed up with serum, or other "indifferent" liquid.

Methylen blue may be fixed in the tissues, and permanent preparations made, by one or other of the methods described in Chap. IX. Bismarck brown stains may be fixed with 0·2 per cent. chromic acid, and the preparations may be stained with safranin, care being taken not to expose them too long to the action of alcohol.

I may say that personally I have found gentian, dahlia, and methylen blue, added to indifferent liquids, extremely useful in the examination of tissue-cells. Quinoleïn and Bismarck brown are well-known aids to the study of Infusoria. Methylen blue has a specific affinity for sensory nerves, and is an extremely important reagent (see *post*, Chap. IX, and Part II).

94. Choice of a Stain.—The following may be recommended with confidence for general work:—For sections, Flemming's method, with safranin or gentian for a single stain, or Henneguy's permanganate modification of the same (Chap. VIII), and the same with hæmatoxylin (§ 106), or Flemming's

orange method (§ 258), or gentian and eosin (Chap. XIII) for a double stain.

For staining *in toto* Grenacher's alcoholic borax-carmine (§ 169), or Mayer's carmalum (§ 151), unless the object be so impermeable as to require a more highly alcoholised stain, in which case take Mayer's para-carmine (§ 168), or for chromic acid objects Mayer's hæmacalcium (§ 182).

For fresh dissociated tissues or small entire objects, methyl green, if it is not important to have permanent preparations; if it is, take alum-carmine.

Picric acid may be used for double staining after carmine or hæmatoxylin.

Many others of the numerous stains discussed in the following chapters render most valuable services, and will be found recommended in the special paragraphs as occasion dictates.

I would add one word of advice to the beginner: never use a double stain where a single one will do. To do so is too often to go farther and fare worse.

And a word of caution to beginners and others:

You are not likely to succeed in staining, especially in the beautiful processes of staining with coal-tar colours, unless you see to it that you are working with chemicals of the proper quality. You *cannot* ensure this by going to a generally trustworthy house for chemical products—at all events, not in the case of coal-tar colours. It is not sufficient that these should be what they are commercially described to be; they may be pure, and yet not give good stains. They must (in the case of anilins, at all events) be the identical products used in their work by the authors who have described and recommended them (see the note on the numerous safranins in the market, § 101). I therefore feel constrained to advise everybody to get his reagents—at all events his anilins—from the well-known chemists Grübler and Münder. Grübler has all the tried reagents in stock, and supplies only such as have been found by experiment with tissues to furnish the desired stain. He also makes up fixing and staining solutions, injection and imbedding masses, &c., according to the classical formulæ, and sends them out neatly packed and ready for use. From experience I can most highly recommend these

preparations, which are in nine cases out of ten better than those the observer is likely to make for himself. They may be ordered from the price list, or by quoting the numbers of the formulæ in this work. The address is: Herrn Dr. G. Grübler, Chemiker, Baiersche Strasse 12, Leipzig. Grübler can correspond in English.

His preparations can be obtained in London from Dr. R. Kanthack, 21, Golden Square, Regent Street, W., who is also the authorised agent for the microscopes and apparatus of Zeiss, also for the microtomes of Becker, and the Bacteriological Apparatus of F. & M. Lautenschlaeger, &c.

Münder's address is: Herrn Dr. G. Münder, Mikroskopisch-chemisches Institut, Göttingen.

Messrs. Squire & Sons, Chemists, 413, Oxford Street, London, W., also make a speciality of microscopical reagents, and I am told furnish excellent products.

CHAPTER VIII.

ANILIN* COLOURS GIVING INDIRECT NUCLEAR STAINS (FLEMMING'S METHOD), AND DIRECT NUCLEAR STAINS.

A. *Indirect Nuclear Stains.*

95. VERY few anilins give a precise nuclear stain by the *direct* method (§ 92). Two of them—methyl green and Bismarck brown—are pre-eminently nuclear stains. Many of the others—for instance, safranin, gentian, and especially dahlia—may be made to give a nuclear stain with fresh tissues by combining them with acetic acid; but in ninety-nine cases out of a hundred are not so suitable for this kind of work as the two colours first named, which practically form a class apart.

Again, very few anilins give a pure plasmatic stain (one leaving nuclei unaffected). The majority give a diffuse stain which in some few cases becomes, by the application of the decoloration or *indirect* method (92), the most precise and splendid stain as yet obtainable by any means.

The indirect staining method, or Flemming's method, and the direct nuclear stains will form the subject of the present chapter, and the remaining anilins will be treated of in the next chapter.

The following list shows the colours treated of in the two chapters.

In Chap. VIII.—A. (*Colours giving indirect nuclear stains,* FLEMMING'S *method.*)—Safranin, gentian violet, dahlia, Victoria blue, anilin green, Magdala red (naphthalin red, rose de naphthaline), mauveïn, rouge fluorescent, solid green, fuchsin (anilin red, rubin, roseïn, magenta, solferino, corallin), orange, Bismarck brown, methyl violet, tropæolin 000, No. 2, (orange II, chysaureïn, β naphthalorange), rocellin (echtroth, or-

* The word "anilin" is here used in the popular sense, to include all coal-tar colours.

seillin No. 3, rubidin, La Rawvarienne), benzoazurin. B. (*Colours giving direct nuclear stains.*)—Methyl green (methanilin green, vert lumière, lichtgrün, grünpulver, vert en cristaux), Bismarck brown (Manchester brown, phenylen brown, vesuvin, La Phénicienne), methyl violet (methylanilin, anilin violet, Paris violet, inchiostro di Leonardi), methyl violet 6 B, fuchsin.

In Chap. IX. — A. Methylen blue. B. (*Plasma and ground stains.*)—Bleu lumière (Parma blue, toluïdin blue, lichtblau), bleu de Lyon (bleu de nuit, Grünstichblau), indulin (nigrosin, bengalin, anilin blue-black, blackley blue, artificial indigo), quinoleïn blue (cyanin, chinolinblau), benzoazurin, anilin blue, violet B., anilin black (blue-black, nigranilin, noir de Colin), Saürefuchsin (fuchsin S., acid fuchsin), Congo red (Congoroth), benzopurpurin, delta purpurin, Biebricher scharlach (Biebrich scarlet), eosin (primerose, erythrosin, pyrosin B., rose B. à l'eau), Bengal rose, picric acid, metanil yellow (metanilgelb), saüregelb (echtgelb), tropæolin O., croceïn, gold orange, iodine green (Hofmann's grün), thiophen green, anilin green, malachite green.

*General Directions for the Indirect or "Flemming" staining Method.**

96. Staining.—*Sections only* can be stained by this method. The solutions employed are made with alcohol, water, or anilin, according to the solubility of the colour. There seems to be no special object in making them with alcohol if water will suffice, the great object being to get *as strong a solution as possible.* The sections must be *very thoroughly* stained in the solution. As a general rule they cannot be left too long in the staining fluid. With the powerful solutions obtained with anilin a few minutes or half an hour will frequently suffice, but to be on the safe side it is frequently well to leave the sections twelve to twenty-four hours in the fluid. Up to a certain point the more the tissues are stained the better do they resist the washing-out process, which is an

* Historically the principle of this method is due to HERMANN and BOETTCHER; but it is universally known by the name of Flemming, to whom is due the credit of having greatly improved the method in its practical details.

advantage. For researches on nuclei the solutions made with anilin had better be employed *only* with preparations well fixed in chromo-aceto-osmic acid, as the basic anilin oil may easily attack chromatin if not specially well fixed.

97. Washing out.—Washing out is generally done with alcohol, sometimes pure, sometimes acidulated (with HCl). The stained sections, if loose (celloidin sections), are brought into a watch-glassful of alcohol; if mounted in series on a slide they are brought into a tube of alcohol (washing out *can* be done by simply pouring alcohol on to the slide, but it is better to use a tube or other bath). It is in either case well to *just rinse* the sections in water before bringing them into alcohol.

The sections in the watch-glass are seen to give up their colour to the alcohol in clouds, which are at first very rapidly formed, afterwards more slowly. The sections on the slide are seen, if the slide be gently lifted above the surface of the alcohol, to be giving off their colour in the shape of rivers running down the glass. In a short time the formation of the clouds or of the rivers is seen to be *on the point of ceasing;* the sections have become *pale* and somewhat *transparent,* and (in the case of chrom-osmium objects) have *changed colour,* owing to the coming into view of the general ground colour of the tissues, from which the stain has now been removed. (Thus chrom-osmium-safranin sections turn from an opaque red to a delicate purple.) At this point the washing out is complete, and *must be stopped instantly.*

It is generally directed that absolute alcohol be taken for washing out. This may be well in some cases, but in general strong (95 per cent.) spirit is found to answer perfectly well.

The hydrochloric acid alcohol process had better only be employed with tissues well fixed with "Flemming," as with tissues imperfectly fixed it may cause swellings. Further, the acid extracts the colour much more quickly from resting nuclei than from kinetic nuclei, which is an advantage or a disadvantage according to the end in view.

As a rough and ready guide to the beginner, it may be stated that washing out should be done with pure alcohol whenever it is desired to have resting nuclei stained as well as dividing nuclei; the other processes serving chiefly to differentiate mitoses.

The length of time necessary for washing out to the precise degree required varies considerably with the nature of the tissues and the details of the process employed; all that can be said is that it generally lies between thirty seconds and two minutes.

In more than one of the methods presently to be described treatment with chromic acid or with iodine forms part of the washing out process. The *rationale* of this is somewhat obscure; the most probable point of view appears to be that the chromic acid acts as a mordant on the chromatin and helps it to retain the stain. It is known on the one hand that chromic acid precipitates safranin from its solutions, so that by admitting a special affinity of chromic acid on the other hand for chromatin, and especially for chromatin in the kinetic state, the explanation is hypothetically complete.

The iodine in Bizzozero's (Gram's) process also appears to act as a fixative of the colour.

It has been ascertained by HENNEGUY that permanganate of potash acts as a mordant for coal-tar colours. For the manner of employing it see § 106.

98. Substitution.—It was stated above that washing out is generally done with alcohol. There exists another mode of washing out that is both of practical importance and of great theoretical interest, one anilin stain may be made to wash out another. Thus methylen blue and gentian violet are discharged from tissues by aqueous solution of vesuvin or of eosin; fuchsin is discharged from tissues by aqueous solution of methylen blue. The second stain "substitutes" itself for the first in the general "ground" of the tissues, leaving, if the operation have been successfully carried out, the nuclei stained with the first stain, the second forming a "contrast" stain. It appears from the interesting paper of RESEGOTTI in *Zeit. f. wiss Mik.*, v, 3, 1888, p. 320, that it may be stated as a very general rule that colours that do *not* give a nuclear stain by the indirect method will wash out those that *do*. Thus he found that—

Safranin, Dahlia, Methyl Violet, Gentian Violet, Rubin, Victoria Blue, Magenta, Basic Fuchsin, are washed out by the following:

Congo, Methyl Green, Iodine Green, Nigrosin, Methylen Blue, Orange, Ponceau, Acid Fuchsin, Aurantia, Cyanin, Eosin, Methylic Eosin, Magdala Red, Bordeaux, Vesuvin.

Resegotti obtained the best results by washing out methyl violet or dahlia with eosin or acid fuchsin (Saürefuchsin).

Flemming has obtained important results by first double-staining sections with safranin followed by gentian violet, and then treating them with concentrated aqueous solution of orange, which, in virtue of its acid qualities, washes out most of the gentian (see the details of the process in the chapter on Combination Stains, § 258).

It has been made out by Kühne (*Centralbl. f. Bakteriol. u. Parasitenk.*, xi, 1892, p. 756; *Journ. Roy. Mic. Soc.*, 1892, p. 708) that saturated or strong solutions of malachite green in anilin oil will wash out fuchsin, methylen blue and crystal violet from sections (the process described by Kühne is one for the staining of bacteria in sections, and I cannot say how far it is applicable to histological ends).

The student will note that the colours in the second of Resegotti's lists may be turned to account for washing out and producing a contrast stain at the same time; he should also take note that this washing out is a true chemical decoloration, and if pushed too far will invade the nuclei as well as the rest of the tissues.

99. Clearing.—The washing out of the colour may be stopped by putting the sections into water; but the general practice is to clear and mount them at once.

You may clear with clove oil, *which will extract some more colour* from the tissues. Or you may clear with an agent that does not attack the stain (cedar oil, begamot oil, xylol, toluol, naphtha, &c.; see the chapter on Clearing Agents). If you have used pure alcohol for washing out, you had perhaps better clear with clove oil, as pure alcohol does not always, if the staining have been very prolonged, extract the colour perfectly from extra-nuclear parts. But if you have not stained very long, and if you have used acidulated alcohol for washing out, clove oil is not necessary, and it may be better not to use it, as it somewhat impairs the brilliancy of the stain. A special property of clove oil is that it helps to differentiate karyokinetic figures, as it decolours resting nuclei more rapidly than those in division.

Some colours are much more sensible to the action of clove oil than others; and much depends on the quality of this much adulterated essence. New clove oil extracts the colour more quickly than old.

Series of sections on slides are conveniently cleared by pouring the clearing agent over them.

When the clearing is accomplished to your satisfaction, mount in damar or balsam, or stop the extraction of the colour if clove oil have been used by putting the sections into some medium that does not affect the stain (xylol, cedar oil, &c.).

100. General Results.—The results depend in great measure on the previous treatment of the tissues. If you have given them a prolonged fixation in Flemming's *strong* chromo-aceto-osmic mixture, and have washed out after staining with acid alcohol and cleared with clove oil, you will get, with some special exceptions, nothing stained but nucleoli and the chromatin of dividing nuclei, that of resting nuclei remaining unstained. If you have given a lighter fixation, with Flemming's weak mixture or some other fixing agent not specially inimical to staining, and have washed out after staining with pure alcohol, you will get the chromatin of resting nuclei stained as well. With some of these stains—Victoria, for instance—it is easy to get cytoplasm stained, in a lighter tone than the nuclei, by merely washing out lightly.

It may be noted here that most of the dyes discussed in this chapter give a stain of a somewhat dead or dull quality, so much so that in some cases chromosomes and nucleoli come out quite opaque. Safranin and anilin green, however, do not do this, but leave the structures beautifully transparent. This is an advantage with thick sections, and sometimes for other reasons; but this transparency of the elements is unfortunately favorable to the production of diffraction lines which may be a hindrance to good definition in delicate work.

101. Safranin—one of the most important of these stains, on account of its great power, brilliancy, and superior permanence in balsam, and also on account of the divers degrees of electivity that it displays for the nuclei and other constituent elements of different tissues.

The great secret of staining with safranin is *to get a good safranin.* It is needful here to insist most urgently on what was said above, § 94, *sub. finem.* Before thinking of working with this important reagent, you should go to Grübler or to Münder and order the safranin you want, specifying whether

you want it for staining nuclei or for staining elastic fibres, or for what other purpose you may require it.

There are presumably at least a score of sorts of safranin in the market, differing to a considerable extent in colour, weight, solubility and histological action. Some are easily soluble in water and not so in alcohol, some the reverse, and some freely soluble in both. Fourteen brands, supplied by Grübler and by Münder, have been studied by Resegotti (*Zeit. f. wiss. Mik.*, v, 3, 1888, p. 320). They all gave positive results with the chromic acid method, to be detailed below; although Grübler had explained that the brands XX, XXBN, TB, had not given positive results (with the usual methods). Resegotti obtained his best results with the brands "Safranin wasserlöslich," "Safranin spirituslöslich," "XX," "XXBN," "TB," furnished by Grübler, and with the brands "Rein," "O," "FII," and "Conc.," supplied by Münder.

Staining.

The majority of safranins are not sufficiently soluble in water, so that solutions in other menstrua must be employed.

A solution much used some time ago is that of Pfitzner (*Morph. Jahrb.*, vi, p. 478, and vii, p. 291), composed of safranin 1 part, absolute alcohol 100 parts, and water 200 parts, the last to be added only after a few days.

The solution of Flemming (*Arch. f. mik. Anat.*, xix, 1881, p. 317) is a concentrated solution in absolute alcohol, diluted with about one half of water.

The solutions of Babes (*Arch. f. mik. Anat.*, 1883, p. 356) are (a) a mixture of equal parts of concentrated alcoholic solution and concentrated aqueous solution (this is very much to be recommended), and (b) a concentrated or supersaturated aqueous solution made with the aid of heat.

Some people still employ simple aqueous solutions.

Lastly, there is the anilin solution of Babes (*Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 470). It consists of water 100 parts, anilin oil 2 parts, and an excess of safranin. The mixture should be warmed to from 60° to 80° C., and filtered through a wet filter. This solution will keep for a month or two.

Zwaardemaker (*Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 212) makes a mixture of about equal parts of alcoholic safranin solution and anilin water (saturated solution of anilin oil in water;—to make it, shake up "anilin oil," which is nothing but pure anilin, with water, and filter).

Any of these stains may be used with any of the following

washing-out processes. Of course you will have to stain longer in the weaker solutions. As to the anilin solutions, see *ante*, § 96.

Washing out and Clearing.

For general directions for washing out and clearing, *see* above, §§ 97 and 99.

Flemming's *first method* (l. c. in last §).—Wash out with pure alcohol, followed by clove oil. This method stains resting chromatin as well as "mitoses."

Flemming's *second method* (*Zeit. f. wiss. Mik.*, i, 3, 1884, p. 350).—Wash out until hardly any more colour comes away, in alcohol acidulated with about 0·5 per cent. of hydrochloric acid, followed by pure alcohol and clove oil. (You may use the HCl in watery solution if you prefer it.) The strength given appears unnecessarily high, and I therefore generally use with good results an alcohol of about 0·2 per cent. of HCl.* (Objects are supposed to have been well fixed—twelve hours at least—in the *strong* chromo-aceto-osmic mixture, and stained for some hours.) Podwyssozki (*Beitr. z. path. Anat. v. Ziegler u. Neuwerk*, i, 1886; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 405) prefers to stain for half an hour only, and wash out with 0·1 per cent of HCl in alcohol. In each of these ways you get "mitoses" and nucleoli alone stained (if the fixation have been performed as above directed).

Flemming's orange-method (very important) is given in the chapter on "Combination Stains" (§ 258).

Podwyssozki (l. c.) gives another method, which consists in washing out (for from a few seconds to two minutes) in a strongly alcoholic solution of picric acid, followed by pure alcohol. Same results (except that the stain will be brownish instead of pure red).

Babes employed for washing out after staining in the aqueous or alcoholic solutions above mentioned, pure alcohol followed by oil of turpentine. For sections stained in the anilin solution he recommends treatment with iodine, according to the method of Gram (see what is said as to the process

* In Flemming's latest work (on the achromatic structures of the cell) he has been using a lower strength than this even, viz. 0·1 per cent. at most, see *Arch. f. mik. Anat.*, xxxvii, 1891, p. 249.

of Gram in the paragraph on gentian violet, *post*, § 102). This process has also been recommended by PRENANT (*Int. Monatsschr. f. Anat.*, &c., iv, 1887, p. 368), who notes that the treatment with the iodine solution should be somewhat longer, and the treatment with alcohol somewhat shorter than with gentian violet sections.

MARTINOTTI and RESEGOTTI (*Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 328) recommend washing out with a freshly prepared mixture of one part of 0·1 per cent. aqueous solution of chromic acid with nine parts of absolute alcohol, followed by pure alcohol and bergamot oil. In my experience this method does not give better results (I think less good) than that of washing out in the simple aqueous solution of chromic acid of Bizzozero followed by alcohol (*see* the paragraph on gentian violet, next §). The latter is certainly a most useful method. It should be mentioned that Martinotti and Resegotti's results refer to lightly stained alcohol-fixed objects, and not to chromo-aceto-osmic objects, which may make a great difference.

GARBINI (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 170) has recommended that sections be dehydrated after staining in methylic alcohol (wood spirit) in which safranin is only very slightly soluble, and decoloured in a mixture of two parts of clove oil with one part of cedar oil. I have not been able to obtain good results by this method.

The reader will remember that safranin may be washed out by substitution (see *ante*, § 98). In preparations made with chromo-aceto-osmic acid, safranin stains, besides nuclei, elastic fibres, the cell-bodies of certain horny epithelia, and the contents of certain gland-cells.

It has been shown by OHLMACHER (*Journ. Amer. Med. Ass.*, vol. xx, No. 5, Feb. 4, 1893, p. 111) that if tissues be treated with solutions containing iodine or picric acid after staining with safranin, there may be produced in the tissue-elements a precipitate of a dark red substance of a crystalline nature but of lanceolate, semi-lunar, falciform or navicellar forms. This precipitate is formed both in normal and pathological tissue, and occurs either in the nuclei or in the cytoplasm. It is formed readily in carcinomatous tissues; and Ohlmacher makes out a strong case in favour of the conclusion to which he has come that many of the bodies that have been described as "coccidia," "sporozoa," or other "parasites" of carcinoma are nothing but particles of this precipitate. This refers, amongst much other work, to that of PODWYSSOZKI and SAWTSCHENKO. Forewarned is forearmed; but if the formation of Ohlmacher's precipitate should prove to be a very general phenomenon, it will be necessary to conclude with him that to follow safranin staining with treatment by solutions containing iodine or picric acid is not only unscientific but positively dangerous. Of course this is not intended to discredit the use of safranin when washed out with hydrochloric acid, alcohol, or the like.

102.—Gentian Violet.—One of the most important of these stains. It may be used in aqueous solution, or in alcoholic solution diluted with about one half of water (FLEMMING,

Zells. Kern. u. Zellth., 1882, p. 384), and the stain may be washed out with pure alcohol or (FLEMMING, *Zeit. f. wiss. Mik.*, 1, 1884, p. 350) with acidulated alcohol, as directed below for safranin. But by far the best way of using it is, in general, that due to BIZZOZERO (*Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 24). The tissues may be hardened either in alcohol or in a chromic mixture, but must in the latter case have been well washed out with water. The staining solution is borrowed from that of EHRLICH for bacteria, and consists of—

Gentian violet	1 part.
Alcohol	15 parts.
Anilin oil	3 „
Water :	80 „

The sections are stained in it for five or ten minutes or longer (for objects from Flemming's solution it will frequently be advisable to stain for as many hours). After staining, rinse the sections with alcohol, and bring them into a 0·1 per cent. aqueous solution of chromic acid. After from thirty to forty seconds bring them into alcohol, which begins the washing out of the colour. After thirty or forty seconds in the alcohol put them back for thirty seconds into the chromic acid (this is done in order to fix the colour more completely in the nuclei). Then bring them back into alcohol for thirty to forty seconds, in order to wash out more colour and dehydrate them at the same time. Then treat with clove oil, which will extract more colour, and after a short time must be changed for fresh, in which the sections remain until they are seen to give up no more colour, when they are removed and mounted in damar.

You might give a longer treatment with alcohol, and a shorter treatment with clove oil, but you would get a slightly different result. Alcohol washes out colour freely from kinetic nuclei as well as from resting nuclei, whereas clove oil acts much more energetically on the latter than on the former, and thus serves to differentiate dividing nuclei.

In some cases, especially those of tissues whose nuclei have a tendency to give up the colour too freely, better results are obtained by combining the foregoing method with that of GRAM for the staining of bacteria (*Fortschr. d. Medicin*, ii, 1884, No. 6; *British Med. Journ.*, Sept. 6th, 1884, p. 486; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 817).

In Gram's method the sections are treated, after staining, with a solution composed of—

Iodine	1 gramme.
Iodide of potassium	2 grammes.
Water	300 „

In Bizzozero's adaptation of this process the series of operations is as follows:—Stain in the gentian, wash for five seconds in alcohol; two minutes in the iodine solution; twenty seconds in alcohol; thirty seconds in the chromic acid solution; fifteen seconds in alcohol; thirty seconds in the chromic acid again; thirty seconds in alcohol; and treatment with changes of clove oil until final decoloration.

Nissen (*Arch. f. mik. Anat.*, 1886, p. 338) employs this process with omission of the treatment with chromic acid.

In resting nuclei the nucleoli alone are stained, or the chromatin if stained is pale; in dividing nuclei the chromatin is stained with great intensity, being nearly black in the equatorial stage.

This exceedingly powerful stain is quite as precise as that of safranin, to which it is perhaps even preferable for much work with very thin sections (thick sections with closely packed nuclei may easily come out too dark). It lends itself admirably to double staining with eosin, with which it affords one of the most useful and beautiful double-stains known (*see* § 258).

The stain keeps fairly well in damar, though not so well as that of safranin. Flemming found that after a year it had fadad a little, though not so much as hæmatoxylin stains (*v. Zells. Kern. u. Zellth.*, p. 384).

Gentian violet in acid solution stains the nuclei of fresh tissues, and dissolved in indifferent media is sometimes very useful for staining *intra vitam* (*see* above, § 93).

Gentian may be washed out by substitution, whence the important method of Flemming, § 258, which *see*.

103. Dahlia (Flemming, *Arch. f. mik. Anat.*, xix, 1881, p. 317).—Stain in an aqueous solution, either neutral or acidified with acetic acid, and wash out with pure alcohol. The stain is paler in the nuclei than with gentian or safranin. The cytoplasmic granulations of certain cells are sharply stained.

Dahlia is also a useful nuclear stain for fresh tissues (v. EHRLICH, *Arch. . mik. Anat.*, xiii, 1876, p. 263). For these the aqueous solution must be acidulated with (7·5 per cent.) acetic acid; or you may stain in a neutral solution, and wash out with acidulated water. Dehydrate with alcohol and mount in turpentine colophonium. It is also useful for staining *intra vitam.* *See* above, § 93.

For the staining of Ehrlich's "plasma cells," *see post*, Part II.

104. Victoria Blue (Victoriablau). (LUSTGARTEN, *Med. Jarhb. k. Ges. d. Aerzte zu Wien*, 1886, pp. 285—91).—Stain (specimens strongly fixed in "Flemming" some hours, lightly fixed specimens a few minutes) in saturated aqueous solution. Wash out in pure alcohol (about one minute, more or less). You may clear with clove oil, but you had perhaps better take cedar or bergamot oil, as clove oil washes out the colour very freely.

A most brilliant and useful nuclear stain, and one that I think should be particularly recommended to the beginner, as it is particularly easy to work with. Chromatin and nucleoli blue. Cytoplasm, if well washed out, colourless; if less washed out, green or greenish blue. The "spongioplasm" is very finely brought out by this method.

Victoria has a special affinity for *elastic fibres.* For this object Lustgarten recommends an alcoholic solution of the dye diluted with two to four parts of water. Fixation in chrom-osmium, or at least in a chromic mixture, is, I believe, a necessary condition to this reaction. And you must stain for a long time.

Victoria has also a special affinity for *mucus-cells*, from which it is not washed out by alcohol.

105. Anilin Green.—Use precisely as directed for Victoria blue, *supra.* An extremely delicate and absolutely precise nuclear stain, nucleoli being peculiarly brilliantly stained by it. I am unfortunately unable to trace the history of the colour used by me, which may be identical with the Solidgrün of Flemming (*Arch. f. mik. Anat.*, xix, 1881, pp. 317 and 742). It is well to be even more careful in the use of clove oil than in the case of Victoria blue. This colour seems not so generally useful as Victoria, as it does not give so bold a stain.

Like Victoria, anilin green has also a special affinity for mucus-cells. *See* the paragraphs on "mucus-cells" in Part II.

106. Henneguy's Permanganate Method (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 397).—This method is based on the fact, discovered by HENNEGUY, that permanganate of potassium is a mordant for many anilin dyes, and will

enable a good stain to be procured in cases in which the usual methods fail.

Sections (from tissues fixed either in the strong solution of Flemming for from two to six hours, or in other reagents such as sublimate, liquid of Perenyi, liquid of Kleinenberg, alcohol, &c.) are treated for five minutes with 1 per cent. solution of permanganate of potassium. They are then washed with water and stained (for about half the time that would have been taken if they had not been mordanted with the permanganate) in safranin, rubin, gentian violet, vesuvin, or the like. The stain that succeeds the best is a safranin solution prepared with anilin water and absolute alcohol (*see* above, § 101). After staining they are washed out with alcohol, followed by clove oil in the usual way. This is the delicate part of the process. The progress of the decoloration must be watched under the microscope, in order that it may be stopped at the proper moment. It goes on in general slowly, and the slower it proceeds the more selective will be the resultant stain. The decoloration sometimes continues even after the sections have been mounted in balsam, especially if all traces of clove oil have not been removed before mounting. It may thus happen that preparations which are insufficiently washed out at the moment of mounting show a perfectly differentiated stain twenty-four or forty-eight hours afterwards.

If safranin have been taken as the stain the preparations show protoplasm of an orange-grey tint, which shows up the most delicate structures, in particular the achromatic figures of cytodieresis. Chromosomes and nuclear membranes are of a brilliant red, attractive spheres and centrosomes less strongly stained, but still sharply brought out against the rest of the cytoplasm.

If it be desired to have the details of protoplasmic structures still more markedly brought out, the safranin stain may be *preceded* by hæmatoxylin staining (0·5 per cent. solution of hæmatoxylin in 90 per cent. alcohol ten minutes, wash with water, treat for ten minutes with 2 per cent. solution of bichromate of potash, and wash with distilled water before treating with the permanganate). But with preparations that have been fixed in Flemming this treatment will hardly be necessary.

The mordanting action of permanganate of potassium on anilin stains is so energetic that if it have been overmuch prolonged before staining with safranin, or, still more, with rubin, it becomes almost impossible to wash out the sections properly; it may be necessary to leave them for nearly a month in clove oil.

Heneguy's preparations are certainly most successful, and I most highly recommend the method for all preparations in which it is desired to have the achromatic cell-structures stained at the same time as the chromatic elements. And I think it may be recommended to all workers who feel the want of a means of slowing the sometimes inconveniently rapid decoloration of sections treated by the Flemming method.

107. Other Nuclear Stains by the Indirect Method.—The foregoing paragraphs nearly exhaust the list of colours giving *good* nuclear stains by the indirect process. Flemming (*Arch. f. mik. Anat.*, xix, 1881, pp. 317 and 742) mentions the following:

Magdala Red (Naphthalin Red, Rose de Naphthaline).—Nearly if not quite as good a stain as any of the foregoing, and superior to all except safranin in respect of permanency. This and the following should, as far as is yet made out, be used in alcoholic solution diluted with about one half of water, and be washed out with pure alcohol, followed by clove oil.

Mauvein and **Rouge Fluorescent** are good stains, but colour some nuclei more deeply than others in the same preparation.

Solid Green (perhaps the same as the anilin green discussed above) is very elective for nucleoli.

Fuchsin (meaning the basic fuchsins, a series of Rosanilin salts having very similar reactions and found in commerce under the names of Fuchsin, Anilin Red, Rubin, Roseïn, Magenta, Solferino, Corallin).—A good but somewhat weak stain, by the alcohol method. Good results are obtained by substitution in the following manner (Graser, *Deutsche Zeit. f. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 378). You either employ the colour as directed for methyl violet (*post*, § 111), or you stain for twelve to twenty-four hours in a dilute aqueous solution, wash out for a short time in alcohol, stain for a few minutes in aqueous solution of methylene blue, and dehydrate with alcohol. A double stain. Chromatin and nucleoli red, all the rest blue.

Ziehl's Carbolic Fuchsin has been recommended as superior to safranin by Schenk (*Ueber Conservirung von Kerntheilungsfiguren*, Bonn, 1890). I do not know where the original formula was published, and take this from *Zeit. f. wiss. Mik.* (vii, 1, 1890, p. 39).

The stain is made either by taking—

Fuchsin	1 gramme,
Acid. carbol. crist. . .	5 grammes,
Alcohol	10 „
Aq. dest.	100 „

or by saturating a 5 per cent. aqueous solution of carbolic acid with concentrated alcoholic solution of fuchsin (the saturation of the carbolic solution with fuchsin is made manifest by the formation of a metallic-looking pellicle on the surface of the liquid). The stain is washed out with alcohol followed by clove oil.

Orange, precise but weak.

Bismarck Brown is not very satisfactory with chromic objects. With alcohol objects it gives a good chromatin stain, but cannot be thoroughly removed from cytoplasm by any means yet discovered.

Kaiser (*Biblioth. Zool.*, H. vii, 1 Hälfte, 1891; *Zeit f. wiss. Mik.*, viii, 3, 1891, p. 363) has obtained good results with Bismarck brown in the following way:—Stain for forty-eight hours, and at a temperature of 60° C., in saturated solution of Bismarck brown in 60 per cent. alcohol (the solution to be made in boiling alcohol), and wash out (until all is decoloured except the karyokinetic figures) in 60 per cent. alcohol, containing 2 per cent. hydrochloric acid or 3 per cent. acetic acid.

To these may be added—

Methyl Violet, perhaps best used according to the method of Resegotti given in § 101; and (according to Griesbach, *Arch. f. mik. Anat.*, xxii, p. 132.

Tropæolin OOO, No. 2 (Orange II; Chrysaureïn, β Naphtolorange), a fine dark orange stain, and

Rocellin (Echtroth, Orseillin No. 3, Rubidin, la Rauvariene), a cherry-red stain.

Benzoazurin has been lately recommended by Martin (see *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 193). Stain for an hour or so in dilute aqueous solution and wash out with HCl alcohol.

B. *Direct Nuclear Stains.*

108. As regards the direct nuclear stains, the reader is reminded that, as was stated in § 95, many if not most of the anilins give a nuclear stain of greater or less purity if they are used in solutions acidified with acetic acid. Under the present heading, only those are mentioned which give in all respects, alike as regards precision and permanence, simplicity of manipulation and other qualities, a really valuable stain. The very exist-

ence of methyl green and Bismarck brown is a sufficient reason for being silent, in this connection, with regard to the rest.

109. Methyl Green.—This is the most common in commerce, of the "anilin" greens. It appears to go by the synonyms of *Methylanilin green, Vert Lumière, Lichtgrün, Grünpulver.* When first studied by Calberla, in 1874 (*Morphol. Jahrb.*, iii, 1887, p. 625), it went by the name of *Vert en cristaux.* It is commonly met with in commerce under the name of more costly greens, especially under that of Iodine green. It is important not to confuse it with the latter, nor with Aldehyde green (*Vert d'Eusèbe*), nor with the phenylated rosanilins, Paris green, and *Vert d'alcali* or *Véridine.*

The chief use of methyl green is as a nuclear stain for *fresh* or *recently fixed tissues.* For this purpose it should be used in the form of a strong aqueous solution containing a little acetic acid (about 1 per cent. in general). *The solutions must always be acid.* (One of the latest books on the subject I have seen gives a formula for a methyl green solution for staining nuclei in which no acid is mentioned. Such a solution *would not give the pure nucleïn reaction which it is the special function of this reagent to produce.*) You may wash out with water (best acidulated) and mount in some acid aqueous medium containing a little of the methyl green in solution. The mounting medium, if aqueous, *must be acidulated.*

Employed in this way, methyl green is a pure nuclear stain, in the sense of being a precise colour-reagent for nucleïn. For *in the nucleus* it stains nothing but the chromosomes, or nucleïn elements : it does not stain either nucleoli of any sort, nor caryoplasm, nor achromatic filaments. *Outside* the nucleus it stains some kinds of cytoplasm and some kinds of formed material, especially glandular secretions (sericin, for instance). But the nucleïn elements are invariably stained of a bright green (with the exception of the nucleïn of the heads of some spermatozoa), whilst extra-nuclear structures are in general stained in tones of blue or violet.

The following paragraph, translated from the paper by Calberla above quoted, appeared inadvertently without comment in the first edition, and has since been repeated without comment in several places.

"He then found that 'the nuclei of subcutaneous connective tissue and

those of vessels and nerve-sheaths stained rose-red, cells of the corium reddish white, and the cells of epidermis greenish blue to pure blue.'"

It should have been added that Calberla here says what he presumably did not mean to say. Methyl green certainly never stains nuclei red, and Calberla's observation should be taken to refer to the results of a double stain with methyl green and eosin, which is mentioned in the somewhat obscurely expressed passage from which the quotation is taken.

Calberla's misstatement is repeated, and made one of the grounds of an important theoretical deduction by Griesbach, in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 365.

Besides being a perfectly pure chromatin stain, methyl green has other advantages. Staining is instantaneous; overstaining never occurs. The solution is very penetrating, kills cells instantly without swelling or other change of form, and preserves their forms for at least some hours, so that it may be considered as a delicate fixative. Osmic acid (0·1 to 1 per cent.) may be added to it, or it may be combined with solution of Ripart and Petit (this, by the way, is an excellent medium for washing out in and mounting in).

Alcoholic solutions may also be used for staining. They also *should be acidulated with acetic acid.*

The stain does not keep easily. It is difficult to mount it satisfactorily in balsam, because the colour does not resist alcohol (unless this be sufficiently charged with the colour); and of preparations mounted with excess of colour in the usual aqueous media the most fortunate only survive for a few months. Dr. Henneguy however writes to me that it keeps well in Brun's glucose medium (*see* § 402).

It was first pointed out, I believe, by Heschl (*Wiener med. Wochenschr.*, 2, 1879), that methyl green is a reagent for amyloid degeneration. His observations were confirmed by Curschmann (*Virchow's Arch.*, t. 79, 1880, p. 556), who showed that it colours amyloid substance of an intense violet, but this (as pointed out by Squire, *Methods and Formulæ, &c.*, Churchill, 1892, p. 37) may be due to its containing methyl violet as an impurity.

Undoubtedly methyl green is one of the most valuable stains yet known. It is the classical nucleïn stain for fresh tissues.

110. Bismarck Brown (Manchester Brown, Phenylen Brown, Vesuvin, La Phénicienne).—A fairly pure nuclear stain that will work either with fresh tissues or with such as have been hardened in chromic acid.

The colour is not very easily soluble in water. You may boil it in water, and filter after a day or two (WEIGERT, in *Arch. f. mik. Anat*, xv, 1878, p. 258). You may add a little acetic or osmic acid to the solution. MAYSEL (*ibid.*, xviii, 1880, pp. 237, 250) dissolves the colour in acetic acid (this solution does not give a permanent stain). Alcoholic solutions may also be used. Paul Mayer recommends a saturated solution in 70 per cent. alcohol; or CALBERLA'S glycerin-and-alcohol mixture, or dilute glycerine (say of 40 per cent. to 50 per cent.) may very advantageously be employed.

The watery solutions must be frequently filtered. The addition to them of carbolic acid has lately been recommended, vide *Journ. Roy. Mic. Soc.*, 1886, p. 908. Bismarck brown stains rapidly, but never overstains. The stain is permanent both in balsam and in glycerin.

The chief use of this colour is for staining objects *in toto*; but it may also be employed for staining sections by the decoloration method (*see* above, § 107).

As has been noted above (§ 93), Bismarck brown has the property of staining certain cellular elements during life (for this purpose it is necessary to see that the colour employed be pure *and neutral*).

111. Methyl Violet (Methylanilin = anilin-violet = Paris violet = inchiostro di Leonardi). — The following process has been recommended by ORTH (*Amer. Mon. Micr. Journ.*, i, 1880, p. 143; *Journ. Roy. Mic. Soc.*, N.S., i, 1881, p. 137): Sections are to be soaked in water, and then brought into the following solution:

Anilin violet	1 part.
Acetic acid	300 parts.

Mount, without washing out, but simply draining, in acetate of potash (acetate 2 parts, water 1 part).

The stain will probably fade within a year or two.

This process does not appear to be of more than very limited applicability. The following, however, due to GRASER (*Deutsche Zeit. f. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 378) may be very generally useful.

Sections are stained for from twelve to twenty-four hours in a (presumably aqueous) solution so dilute that at the end of that time the sections will have taken up all the colour from the liquid. They are then washed out for a short time in acidulated alcohol, and then in pure alcohol (followed presumably by clearing and mounting in balsam). Schiefferdecker, whose account is here quoted, says that the results, as regards nuclear figures, are

even finer than with safranin. The method is applicable to objects fixed in "Flemming."

A useful stain for fresh tissues is also obtained by using dilute acetic acid in the manner recommended above (by ERHLICH) for Dahlia, § 103.

Methyl violet 6 B has been recommended by KROMAYER for demonstrating the fibrillar structure of epithelium, see Part II, "*Epithelium.*"

112. Amyloid matter appears red in preparations stained with methyl violet. This appears to be an optical effect, see the curious experiments of CAPPERELLI, in *Archivio per le scienze mediche*, iii, No. 21, p. 1.

CHAPTER IX.

METHYLEN BLUE, AND OTHER ANILINS.

A. *Methylen Blue.*

113. The Uses of Methylen Blue.—This colour seems to be in a fair way to become one of the most important reagents in the histological laboratory. Its importance as a stain for micro-organisms in tissues is well known to all pathologists.

As a histological reagent, it is used for sections of hardened central nervous tissue, in which it gives a specific stain of medullated nerves (*post,* Part II). It is a valuable specific reagent for plasma-cells (for which see also Part II). It possesses the property of washing out the stain of certain other anilins, with which it gives valuable double-stains (*post,* Chapter XIII). It stains a large number of tissues *intra vitam,* with little or no interference with their vital functions. And last, not least, it can be made to furnish stains of nerve-tissue, intercellular cement-substances, lymph-spaces, and the like, that are essentially identical with those furnished by a successful impregnation with gold or silver. The results are quite equal in most cases to those of gold or silver impregnation, and are obtained with far greater ease and certainty. I call especial attention to these processes, which seem to be effecting a revolution in histological technique.

114. Staining *in toto* during Life.—Small and permeable aquatic organisms may be stained during life by adding to the water in which they are confined enough methylen blue to give a scarcely perceptible or at least very light tint to the water. If transparent organisms be taken, they may be examined alive without further manipulation at any desired moment. Microscopically examined, they will be found after a time to be partially stained—that is, it will be found that certain tissues have taken up the colour, others remaining

colourless. If now you put back the animals into the dilute solution and wait, you will find on examination after a sufficient lapse of time that further groups of tissues have become stained. Thus it was found by EHRLICH (*Abh. k. Akad. Wiss. Berlin,* February 25, 1885) to whom the principle of the methods under consideration is due, that on injection of the colour into living animals axis cylinders of *sensory* nerves stain, whilst *motor* nerves remain colourless. The motor nerves, however, will also stain, though later than the sensory nerves. It might be supposed that by continuing the staining for a sufficient time, a point would be arrived at at which all the tissues would be found to be stained. This, however, is generally not the case. It is always found that the stained tissues only keep the colour that they have taken up for a short time after they have attained the maximum degree of coloration of which they are susceptible; as soon as that point is attained they begin to discharge the colour even more quickly than they took it up. And it is very often found that the elements which have stained first will have lost much or all of their colour by the time that those which stain later have attained their maximum coloration. It may even happen, as I have observed, that the whole of the stainable tissues of an animal may run through the total gamut of coloration and decoloration until the animal has become as colourless as when first put into the tinted water, and that without any apparent change in its vital activities.

It follows that a total stain of all the tissues of an organism can hardly be obtained under these conditions, but that a specific stain of one group or another of elements may be obtained in one of two ways. If the tissue to be studied be one that stains earlier than the others, it may be observed during life at the period at which it alone has attained the desired intensity of coloration, and the remaining tissues are not yet coloured at all, or not coloured enough to be an obstacle to observation. If it be one that stains later than the others, it may be studied during life at the period at which the earlier stained elements have already passed their point of maximum coloration and have become sufficiently decoloured not to be an obstacle to the observation of the later stained ones; the latter being either at the point of maximum coloration or at a point of desired intensity either

earlier or later than the maximum. Or the observer may fix the stain in either of these stages and preserve it for leisurely study by means of one of the processes given below under the heading "*Preservation of the Preparations.*"

The proper strength of the very dilute solutions to be employed in the manner here considered must be made out by experiment for each object. I think the tint is practically a sufficient guide, but it may be stated that when in doubt a strength of 1·100000 may be taken, and increased or diminished as occasion may seem to require. Zoja (*Rendic. R. Ist. Lombardo,* xxv, 1892; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 208) finds that for Hydra the right strength is from 1·20000 to 1·10000.

The stain is somewhat capricious. It is not possible to predict without trial which tissues will stain first in any organism. It is to be remarked that the stain penetrates very badly, which is perhaps the chief cause of its seeming capriciousness, and, I take it, the chief determining condition of the order in which tissues stain. Gland-cells generally stain early; then, in no definable order, other epithelium-cells, fat-cells, plasma-cells, 'Mastzellen,' blood and lymph corpuscles, elastic fibres, smooth muscle, striated muscle. There are other elements that stain in the living state, but not when the staining is performed by simple immersion of intact animals in a dilute staining solution in the manner we are considering. Chief amongst these are nerve-fibres and ganglion-cells, which remain unstained in the intact organism, most likely, so far as I can see, for the simple reason that the stain is not able to penetrate them.

115. Staining Nerve-tissue during Life.—It is commonly believed that methylen-blue stains of the class we are considering are the product of a *vital* reaction of the tissues, and cannot be obtained with dead tissue. It would, however, appear to be more correct to say that methylen blue has the property of staining living tissue, not because of its being living, but in spite of its being living. Dogiel (*Arch. f. mik. Anat.*, xxxv, 1890, pp. 305 *et seq.*) found that muscle-nerves of limbs of the frog could be stained as much as from three to eight days after the limbs had been removed from the animal. He concludes, indeed, that the reaction shows that

the nerves were still living at that time. But it seems more natural to conclude with APÀTHY (*Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 15 *et seqq.*) that nerve-tissue can be stained after life has ceased.

APÀTHY has directly experimented on this point, and sums up the necessary conditions as follows :—The tissue need not be living, but must be fresh; nothing must have been extracted from it chemically, and its natural state must not have been essentially changed by physical means. For example, the tissue must not have been treated with even dilute glycerin, nor with alcohol, though a treatment for a short time with physiological salt solution is not very hurtful; it must not have been coagulated by heat.

Another common belief concerning the methylen-blue nerve-reaction is that the presence of oxygen is necessary to the reaction. It is therefore the usual practice to dissect out the organ to be investigated after having exposed it to the action of methylen blue by injection or immersion, and leave it for some time exposed to the air. APÀTHY has also investigated this point, and finds (loc. cit., p. 25) that the practice is in some cases correct, but the belief erroneous. It has been explained above that shortly after a tissue has attained the maximum degree of coloration of which it is susceptible it begins to give up its colour again to the surrounding liquid. The larger the volume of liquid with which the tissue is surrounded, the faster will this washing-out process go on; and in order that it may not go on with excessive rapidity, washing out the stain from the nerve-fibres as well as from the earlier stained elements (which alone it is desired to wash out, so as to leave a differentiated specific stain of nervous elements), it is in many cases desirable to have the process go on in presence of as little liquid as possible. Another consideration that justifies the practice is that by exposure to air the preparations take up a trace of *ammonia* (derived from the ammonium carbonate of the air); and Apàthy has experimentally established that this is an important factor in the sharpness of the stain. Oxygen has nothing to do with it.

116. Staining Nerve-tissue by Injection or Immersion.—The practice of the earlier workers at this subject was (following

Ehrlich) to inject methylen blue into the vascular system or body-cavity of a living animal, wait a sufficient time for it to take effect on the organ to be stained, then remove the organ for further preparation and study. And there appears to have been a belief with some workers that it was an essential or at least a desirable condition to the production of the stain that it should have been brought about by injection of the colouring matter into the entire animal. It is now known that this is immaterial, and that the reaction can equally well be obtained by removing the organ and subjecting it to a bath of the colouring matter in the usual way. So that treatment with the colour by means of injection or by means of immersion in a solution may be taken to be a matter of convenience only.

117. The Solutions employed.—The solutions used for injection are generally made in salt solution (physiological, or a little weaker); those used for staining by immersion are made either in salt solution or other "indifferent" liquid, or in pure water. Very various strengths of solution have been employed. The earlier workers generally took concentrated solutions. Thus Arnstein (*Anat. Anz.*, 1887, p. 125) injected 1 c.c. of saturated solution into the vena cutanea magna of frogs, and removed the organ to be investigated after the lapse of an hour. Biedermann (*Sitzb. d. k. Akad. Wiss. Wien, Math. Nat. Cl.*, 1888, p. 8) injected 0·5 to 1 c.c. of a nearly saturated solution in 0·6 per cent. salt solution into the thorax of crayfishes, and left the animals for from two to four hours before killing them. Mayer (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 423) took a strength of 1 : 300 or 400 of 0·5 per cent. salt solution. This can be introduced into the system either by means of a syringe or other injecting apparatus, or by auto-injection through the heart. Even rabbits support this operation if artificial respiration be maintained. The solutions of Retzius are of the same strength. But the tendency of more recent practice is decidedly towards the employment of weaker solutions. Apàthy (*Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 25, 26, *et seqq.*) finds that it is not only superfluous, but positively disadvantageous, to take solutions stronger than 1 : 1000.

For staining by immersion similar solutions to those used

for injecting may be employed, but they should, if anything, be still weaker. Dogiel (*Arch. f. mik. Anat.*, xxxv, 1890, p. 305; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 509) places objects in a few drops of aqueous or vitreous humour, to which are added two or three drops of a $\frac{1}{16}$ to $\frac{1}{15}$ per cent. solution of methylen blue in physiological salt solution, and exposes them therein to the air. In thin pieces of tissue the stain begins to take effect in five or ten minutes, and attains its maximum in from fifteen to twenty minutes. For thicker specimens—retina, for instance—several hours may be necessary, the preparation being kept just moist by occasional treatment with a drop or two of indifferent liquid or methylen-blue solution, added by turns.

118. Apàthy's **Methods.**—As a good example of this kind of work, I subjoin a short account of the procedure recommended by Apàthy (*Zeit. f. wiss. Mik.*, ix., 1, 1892, p. 15) for *Hirudinea*. A portion of the ventral cord is exposed, and if it be considered desirable, dissected out, but the sinus and pigmented connective tissue around it had better not be removed till the staining and fixation are completed. If, however, it be desired to stain as many ganglion-cells as possible as well as fibres, the lateral nerves, as well as the connectives, should be cut through near a ganglion. The preparation is then treated with the stain. This is, for the demonstration chiefly of fibres in *Hirudo* and *Pontobdella*, either a 1 : 1000 solution in 0·5 to 0·75 per cent. salt solution, allowed to act for ten minutes; or a 1 : 10,000 solution allowed to act for an hour to an hour and a half; or a 1 : 100,000 solution allowed to act for three hours (*Lumbricus* requires twice these times; *Astacus* and *Unio* require three times; medullated nerves of Vertebrates four times). For the demonstration of ganglion-cells the stain is allowed to act three or four times as long (the connectives and lateral nerves having been cut as directed above).

The staining having been accomplished, the preparations from the 1 : 1000 solution are washed in salt solution for an hour; those from the 1 : 10,000 solution for a quarter of an hour; those from the 1 : 100,000 solution need not be washed at all. They are then treated with one of the ammoniacal fixing and differentiating liquids described below in the

paragraph on "Preservation of the Preparations" (§ 119). This is done by pouring the liquid over them, and leaving them in it *without moving them about in it* for at least an hour, and by preference in the dark. The further treatment is as described in § 119.

The object of the ammonia in these liquids is to *differentiate* the stain—to produce an artificial "secondary differentiation." It acts by washing out the absorbed colour from certain elements, others resisting its action longer, much as HCl alcohol washes out a borax-carmine stain. In this case the elements that are washed out are the protoplasmic parts of nerve-fibres, and their "interfibrillar" and "perifibrillar" substance, the "primitive fibrils" still retaining the colour strongly. It is of theoretical interest to remark that according to Apàthy the coloration thus obtained is a true *stain* of the "primitive fibrils," not an *impregnation*. The "primitive fibrils" are sharply stained of a violet-blue, showing no granular precipitate, and the "interfibrillar" and "perifibrillar" substance, as well as nuclei, are either colourless or very lightly stained. The usual methods, on the other hand, give an "inverse" reaction, the "primitive fibrils" remaining colourless, whilst the interfibrillar substance and protoplasm of the nerve-fibres are *impregnated* with a finely granular greenish-black or violet precipitate, and the nuclei are usually stained.

119. Preservation of the Preparations.—There are considerable difficulties in the way of obtaining permanent preparations of methylen-blue stains, as the stain is so very unstable that, as above explained, it begins to discharge after a short time, even in the living and not yet totally impregnated tissue. The colour may, however, be fixed, and more or less permanent preparations be made, by one or other of the following methods:

Dogiel (*Arch. f. mik. Anat.*, xxxiii, 4, 1889, pp. 440 *et seq.*), following Arnstein (*Anat. Anzeig.*, 1887, p. 551), brings the preparations, in order to fix the colour, into saturated aqueous solution of picrate of ammonia, in which they are allowed to remain for half an hour or more, and are then removed, washed in fresh picrate of ammonia solution, and studied in dilute glycerin, or mounted permanently in glycerin saturated

with picrate of ammonia. More recently (*Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 15) he has recommended an increased duration of the picrate of ammonia bath up to eighteen or twenty-four hours, and mounting, without washing out, in chemically pure glycerin, free from acid. There is a defect in this process, namely, that picrate of ammonia has a very injurious action of a macerating nature on some tissues. This may, however, be avoided by adding to the fixing-bath 1 to 2 per cent. of a 1 per cent. osmic acid solution. (If it be desired to harden the tissues so that sections may be cut, the proportion of osmium solution should be increased fourfold.)

S. Mayer (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 422) preferred a mixture of equal parts of glycerin and saturated picrate of ammonia solution, which served to fix the colour and mount the preparations in. This was also in principle the method followed by Retzius (*Intern. Monatsschr. Anat. u. Phys.*, Bd. vii, H. 8, 1890). Dogiel, after careful study, quite refuses to admit that this is in any way an improvement.

Other workers have employed saturated solution of iodine in iodide of potassium (so Arnstein), or picro-carmine (so Feist, *Arch. f. Anat. u. Entwickel.*, 1890, p. 116; cf. *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 231), the latter having the advantage of preserving the true blue of the stain if it be not allowed to act too long, and the preparation be mounted in pure glycerin.

Picric acid has been used by Lavdowsky, but this, too, after careful study, is rejected by Dogiel.

Apàthy (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 30) has found, as stated above, that free ammonia is a capital factor in the differentiation of the stain. He brings preparations (after washing in salt solution if the staining have been performed with a strong methylen-blue solution, or without washing if it have been done with a very dilute solution) either into a concentrated aqueous solution of picrate of ammonia *free from picric acid*, and containing five drops of concentrated ammonia for every 100 c.c.; or, which is generally preferable, into a 1 to 2 per cent. freshly prepared solution of neutral carbonate of ammonia saturated with picrate. They remain in either of these solutions, preferably in the dark, for *at least an hour*. They are then brought into a *small* quantity of saturated solution of picrate of ammonia in 50 per cent.

glycerin, where they remain until thoroughly saturated. They are then removed into a saturated solution of the picrate in a mixture of 2 parts 50 per cent. glycerin, 1 part cold saturated sugar solution, and 1 part similarly prepared gum-arabic solution. When thoroughly penetrated with this they are removed and mounted in the following gum-syrup medium (l. c., p. 37) :

Picked gum-arabic . . .	50 grms.
Cane-sugar (not candied) . .	50 „
Distilled water	50 „

Dissolve over a water-bath and add 0·05 grm. thymol. This mounting medium sets quickly and as hard as balsam, so that no cementing of the mounts is necessary. Farrant's medium (with omission of the arsenious acid) will also do. In neither case should either ammonium picrate or methylen blue be added to the medium.

None of the preceding methods can be said to be anything like perfectly satisfactory. The stain is generally not preserved in its true blue colour, but turns to a grey, varying in tone from reddish brown to bluish or greenish black. The preparations seldom keep even in that state for more than a very few months, and it is not satisfactory to be obliged to mount preparations only in aqueous media. A strong solution of platinum chloride is said to give a fixation that will resist the treatment necessary for imbedding either in celloidin or paraffin (see Feist, *Arch. f. Anat. und Entw.*, 1890, p. 116; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 231) ; but the precipitate it gives is a flocculent one, and the preparations are not very satisfactory.

The method of Parker (*Zool. Anzeig.*, No. 403, 1892, p. 375) is therefore a most welcome step forwards. The stain is fixed (1) (in the form of a finely grained purplish precipitate) by cold concentrated aqueous solution of corrosive sublimate. The preparations are dehydrated (2) in a solution composed of 1 grm. of sublimate and 5 c.c. of *methylal* (pure methylal washes out the stain to a certain extent). The methylal solution is now removed by means of a mixture (3) of two parts xylol, one part pure methylal, and one part of the dehydrating mixture (2). After a short time the preparations are placed in (4) a considerable quantity of xylol. Here they should remain till all the methylal is replaced by xylol and the corrosive sublimate completely washed out. This will

take some four or five days, as sublimate is very little soluble in xylol. Mount in balsam, or if sections be desired, imbed in paraffin in the usual way. Sections should be fixed to the slide with Schällibaum's collodion, and not with Mayer's albumen, which discharges the colour. The preparations will keep for several weeks, but the finer details are likely to fade after a month.

The times required for a ganglion of the ventral chain of a crayfish are for (1) ten minutes, (2) fifteen minutes, (3) ten minutes, (4) four or five days.

Feist, in the paper quoted above, recommends Joliet's gum-glycerin imbedding method; but in most cases the method of Parker will doubtless be preferable.

Preparations preserved by the old methods (I do not know whether it is the case with preparations preserved by Parker's method) are extremely sensitive to the influence of light. Diffused daylight is less injurious to them than the light concentrated on them by the illuminating apparatus of the microscope during observation. Apàthy finds that lamplight is particularly injurious, especially the intense lamplight used with high powers; which he attributes partly to the yellow rays, partly to the heat.

120. Methylen-Blue Impregnation of Epithelia, Lymph-spaces, &c. (Dogiel, *Arch. f. mik. Anat.*, xxxiii, 4, 1889, p. 440, *et seq.*).—Suitable pieces of tissue (thin membranes by preference) are brought fresh into a 4 per cent. solution of methylen blue in physiological salt solution. After a few minutes therein they are brought into saturated solution of picrate of ammonia, soaked therein for half an hour or more, then washed in fresh picrate of ammonia solution, and examined in dilute glycerin.

If it be wished only to demonstrate the outlines of endothelium cells, the bath in the stain should be a short one, not longer than ten minutes in general; whilst if it be desired to obtain an impregnation of ground-substance of tissue so as to have a negative image of juice-canals or other spaces, the staining should be prolonged to fifteen or thirty minutes, and it is advisable to remove the endothelial covering of the objects operated on before putting them into the stain.

If it be desired to preserve the preparations permanently, they had better be mounted in glycerin saturated with picrate of ammonia. (For an improvement in the method of preservation given in a later paper see *supra*, § 119.)

The effect is practically identical (except as regards the colour) with that of a negative impregnation with silver nitrate.

121. Mayer's (S.) Impregnation Methods (*Zeit. f. wiss.*

Mik., vi, 4, 1889, p. 422).—Mayer's experiments were made contemporaneously with those of Dogiel, cover much the same ground, and give the same results. Mayer stained tissues for about ten minutes in a 1 : 300 or 400 solution of methylen blue in 0·5 per cent. salt solution, rinsed in salt solution, and put up in the glycerin-picrate of ammonia mixture given above, § 119. He finds it may be stated that by this method *all the essential results of a nitrate of silver impregnation may be produced by means of methylen blue.* The images are either positive or negative. In stratified epithelia and in endothelia (for instance, in the testicular canals of the rat, in the vascular system, in smooth muscle) cement-substance is stained. In the cornea, ground-substance is stained, giving a negative image of the corneal corpuscles; and in like manner a negative image is sometimes obtained of the intra-sarcolemmar nerve-endings in striated muscle of frogs and rats. But methylen blue sometimes, as does sometimes silver nitrate, gives positive images of the cornea. If the stain be brought about by injection of the colour into the vascular system, the positive impregnation is the more frequent; whilst if it have been brought about by the immersion of the cornea, a negative image is more frequently obtained. In medullated nerves, striking images of the cruciform figures in the constrictions of Ranvier are obtained, just as with silver nitrate.

To sum up, almost any reaction that can be obtained with chloride of gold, or with nitrate of silver, can be obtained with methylen blue, and with much greater ease and certainty.

122. Other Uses of Methylen Blue.—Methylen blue is also used, chiefly in conjunction with other colouring matters, for staining fixed and hardened tissues, especially preparations of central nervous system. These uses will be described in their proper places.

B. *Other Anilins (Plasma and Ground Stains).*

123. Bleu Lumière is stated to be a plasma stain not affecting nuclei. I have not been able to make out whether it is identical with **Parma blue,** which is one of the numerous toluidin blues. If it is, Frey recommends a solution in water of 1 : 1000, in which tissues stain in a few minutes, and

may be mounted either in glycerin or balsam. "**Lichtblau**" is possibly a synonym of this colour. The principal use of such a colour is for making double stains.

123a. Carmine Blue (Bleu Carmin Aqueux).—This colour is much recommended by GILSON, into whose laboratory it was introduced by JANSSENS. See *La Cellule*, vii, 1891, p. 347.

124. Bleu de Lyon (Bleu de Nuit, Grünstichblau).—I quote this colour here, although I am not sure to what extent it is a pure plasmatic stain. It is said to be very useful for double-staining with carmine.

125. Indulin (Nigrosin, Bengalin, Anilin Blue-black, Blackley Blue, Artificial Indigo). (Introduced by Calberla, see *Morph. Jahrb.*, iii, 1877, p. 627.)—Indulin dissolves into a dark blue solution in warm water or in dilute alcohol. For staining, the concentrated aqueous solution should be diluted with six volumes of water. Sections will stain in the dilute solution in five to twenty minutes; they may be washed in water or in alcohol, and examined either in glycerin or oil of cloves.

The peculiarity of this stain is that it never stains nuclei; the remaining cell-contents and intercellular substance are stained blue. In its general effects it resembles quinoleïn blue, and is exactly the opposite of methyl green. The stroma of tendinous tissue, for instance, stains of a fine blue, the connective tissue that surrounds the bundle hardly at all, and the tendon-corpuscles of Ranvier remaining perfectly colourless, stand out as white stellate figures on a blue ground.

126 Quinoleïn Blue (Cyanin, Chinolinblau; v. Ranvier, *Traité*, p. 102).—Quinoleïn should be dissolved in alcohol of 36° strength (*i. e.* 90 per cent.), and the solution diluted with an equal volume of water. (If the alcohol were taken dilute in the first instance the blue would not dissolve.) The solutions employed for staining should be very weak, as quinoleïn stains very powerfully.

After staining, wash and mount in glycerin. When first mounted, nuclei will be seen to be stained a fine violet, nerves of a grey-blue, smooth muscle blue, protoplasm blue, fat deep blue. But after twenty-four hours in the glycerin the aspect of the preparation is changed; the nuclei have become colourless; the protoplasm remains blue, and is seen to contain granulations stained intensely blue; nerves remain grey-blue, but frequently contain granulations stained blue. Quinoleïn, in a word, has the property of staining fatty matters an intense blue.

If the stained preparations be treated with solution of potash of 40 per cent. strength, the differential reaction is produced immediately; the nuclei are unstained, protoplasm, nerve, and muscle-tissue are pale blue, and fatty matters deep blue.

Quinoleïn is useful for staining Infusoria, which in dilute solution it stains during life. On this point see the methods of Certes (*post*, Part II).

127. Benzoazurin may be made to give either a diffuse or a

nuclear stain, according to MARTIN (see *Journ. Roy. Mic. Soc.*, 1890, p. 114).

128. Anilin Blue.—Used alone, without special precautions, this is a diffuse stain hardly to be recommended at the present time. See, however, the method of HEIDENHAIN, *Arch. f. mik. Anat.*, vi, 1870, p. 404. It is useful for double-staining.

129. Violet B. (S. MAYER, *Sitzb. d. k. k. Akad. d. Wiss., Wien*, iii Abth., February, 1882).—Used in solutions of 1 grm. of the colour to 300 grms. of 0·5 per cent. salt solution, and with fresh tissues that have not been treated with any reagent whatever, this colour gives a stain so selective of the elements of the vascular system that favorable objects, such as serous membranes, appear as if injected. The preparations do not keep well; acetate of potash is the least unsatisfactory medium for mounting them in.

130. Anilin Black (Blue-black, Nigranilin, Noir de Colin).—Has a special affinity for ganglion-cells, and is much used in the study of the central nervous system. See *post*, Part II.

According to CERTES, anilin black dissolved in sea or fresh water is useful for the study of Infusoria, to which it is in no way toxic.

131. Säurefuchsin (Fuchsin S., Acid Fuchsin).—A fuchsin in which the colouring principle is an acid, instead of being a base as in ordinary fuchsin. It is only made by the "Badische Anilin und Soda Fabrik." It may be obtained from Grübler or the other providers of histological reagents. It is a powerful diffuse stain, having a special affinity for axis-cylinders, and is chiefly used for staining nerve-centres. See the chapter on Nerve-Centres, in Part II.

132. Congo Red (Congoroth) (see Griesbach, in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 379).—Also an "acid" colour, in the sense in which that appellative is given to Säurefuchsin. The aqueous solution, however, has a neutral or alkaline reaction. It becomes blue in presence of the least trace of free acid (hence Congo is a valuable reagent for demonstrating the presence of free acid in tissues; see the papers quoted, l. c.). A diffuse stain, much of the same nature as that of Säurefuchsin, and like it seems to be at present chiefly useful in staining axis-cylinders. See the chapter on Nerve-Tissue, in Part II. It may also be used for staining some objects during life. See *ante*, § 93.

133. Benzopurpurin.—According to Griesbach (l. c.), another "acid" colour very similar in its results to Congo red. It is probably one of the best diffuse stains yet made known, and as a contrast stain to hæmatoxylin, will very likely take the place of eosin in many cases. It is a very strong stain, therefore a *very* weak solution should be taken, and only allowed to act for a minute or so. ZSCHOKKE (ibid., v, 4,

1888, p. 466) says that **Benzopurpurin B.** is the very best contrast stain to hæmatoxylin known to him. It seems to him preferable to eosin on account of its not being affected by alcohol or the usual clearing agents. Weak aqueous solutions should be used for staining, which is effected in a few minutes, and alcohol for washing out. **Deltapurpurin**, a more purple red, has similar properties, and may be used in the same way. The solution (aqueous) should be moderately concentrated, and allowed to act for a minute or two.

134. Biebricher Scharlach (BIEBRICH SCARLET), a diffuse bright red stain, may be useful as a contrast stain. See Griesbach, *Arch. f. mik. Anat.*, xxii, p. 132.

135. Eosin, the potassium salt of a bromide of phthaleïn, is found in commerce under the synonyms of **Primerose Soluble, Erythrosin, Pyrosin B., Rose B. à l'Eau.** The preparations indicated by these names are not quite identical in their properties, but vary according to the different modes of manufacture. Most of them are soluble both in alcohol and in water, but some only in alcohol (*"Primerose à l'Alcohol"*).

Eosin is of very great importance as a secondary or contrast stain. It has already been mentioned that it washes out many of the anilins that give nuclear stains by the indirect method (see § 98). Combined with a blue anilin, or with hæmatoxylin, it gives instructive and durable double stains, which are among the most beautiful that can be produced. These will be treated of in the chapter on double stains. Here it may merely be mentioned that the majority of the authors who recommend these combinations proceed by staining first with the eosin and then with the nuclear stain, and that the majority also employ alcoholic solutions of eosin. I am by no means convinced that this practice is the best. As regards the anilins, at all events, good results are certainly obtained by staining first with the nuclear stain (gentian violet, for instance) and washing out (sections) for a few seconds or a minute or two in a tolerably strong aqueous solution of eosin, and then dehydrating with alcohol and mounting in balsam. If the eosin is too much extracted by the alcohol or clearing agent, these should be used charged with a little of the colour. Eosin stains may also be kept in glycerin, if this be perfectly neutral or, better, slightly alkaline (which may

be brought about by the addition of 1 per cent. of sodium chloride), and also charged with a little eosin.

136. Bengal Rose (GRIESBACH, *Zool. Anz.*, No. 135, 1883, p. 172).—Bengal rose, or "Rose bengale," or "Bengal rosa," is an eosin dye. It is the bluest of the eosin dyes as yet known, approaching in hue to fuchsin, but possessing far greater brilliancy and purity of hue. In aqueous solution it is useful for staining chromic acid objects, especially spinal cord, in which the grey matter stains of a deep bluish red, and stands out boldly from the less deeply coloured white matter. It is also useful for double and treble stains, as will be explained below.

137. Picric Acid.—Not used alone as a stain, but one of the most useful of all colouring agents as a secondary or ground-stain. Nothing is easier than to stain with an alcoholic solution of picric acid tissues of which the nuclei have previously been stained by borax-carmine, alum-carmine, hæmatoxylin, or an anilin stain. It should be borne in mind that picric acid has considerable power of washing out other anilin stains; and that in combination with hydrochloric acid it very greatly enhances the power with which this acid washes out carmine stains. It does not otherwise affect any of the usual stains, and may be most highly recommended as a useful though frequently inelegant stain.

138. Metanil Yellow (Metanilgelb).—This colour has lately been studied with great minuteness by GRIESBACH (*Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 448; see also *Journ. Roy. Mic. Soc.*, 1889, p. 464). It does not appear to be worthy of having so much time spent on it from the practical point of view, the interest of Griesbach's work lying rather in the region of chemical theory. The practical outcome is that metanil yellow is a diffuse stain with a certain affinity for various elements belonging to the group of the connective tissues, and that with some other colours it gives sharply differentiated double stains of certain preparations. These will be mentioned in the proper places.

139. Säuregelb (Echtgelb), Tropæolin O., Croceïn, Gold Orange, are all of them more or less diffuse yellow or orange stains, having certain affinities for certain tissues, and may occasionally be found very useful for double-staining, being good stains in their way (see GRIESBACH, *Arch. f. mik. Anat.*, xxii, p. 132).

140. Iodine Green ("**Hofmann's Grün**"), see GRIESBACH, *Zool. Anz.*, No. 117, vol. v, 1882, p. 406.—The high praise accorded to this stain by Griesbach has not been justified by the experience of other workers. The colour is now no longer

manufactured for industrial purposes, but may be obtained of excellent quality from C. A. F. Kuhlbaum's Chemische Fabrik, Berlin, S.O. (*Zool. Anz.*, No. 130, 1883, p. 56).

141. Thiophen Green (**Thiophengrün**), see KRAUSE, *Intern. Monatsschr. f. Anat.*, &c., iv, 1887, Heft 2.

142. Anilin Green has a special affinity for mucous gland cells. We shall return to this subject in the chapter on glands.

143. Malachite Green is a colour concerning which I have not been able to obtain sufficient information. It appears to be useful as a plasma-stain.

CHAPTER X.

CARMINE AND COCHINEAL STAINS.

144. The Theory of Carmine Staining.—I take the following from the important paper of MAYER, "Ueber das Färben mit Carmin, Cochenille, und Hämateïn-Thonerde," in *Mitth. a. d. Zool. Station zu Neapel*, Bd. x, Heft 3, 1892, p. 480. The *rationale* of staining with carmine has hitherto been obscured by the erroneous notion that carmine is nothing but carminic acid with at most certain impurities. This is not the case. According to the analysis of LIEBERMANN (Ber. d. Chem. Ges., Jahrg. 18, 1886, pp. 1969—1975) carmine is a *very peculiar alumina-lime-protein compound of carminic acid*, a true chemical compound from which at all events *aluminium* and *calcium* can no more be absent than sodium from salt. Analysis gives about 17 per cent. of water, 20 per cent. nitrogenous matters, 56 per cent. carminic acid, at least 3 per cent. alumina, and 3 per cent. lime, together with a small proportion of magnesia, potash, soda, phosphoric acid, and a trace of wax. Mayer has come to the conclusion that in the processes of histological staining (*not* of industrial dyeing) the active factors of the compound are, besides the carminic acid, only the alumina, and, in some cases, the lime. The other bases are inactive; the nitrogenous matters, so far as they have any influence at all, are an obstacle, as it is they that give rise to the well-known putrefaction of the solutions.

Having arrived at these conclusions, it seemed logical to admit that *carminic acid, instead of carmine, should be taken as the basis of staining solutions.* This had already been proposed by DIMMOCK, whose paper (*Amer. Natural.*, xviii, 1884, pp. 324–7) I quoted at length in the first edition of this work. But Dimmock's proposals were not very successful, for the reason that he had omitted from his solutions the essential element, the alumina. He stained, for instance, with pure

alcoholic solution of carminic acid, or of carminate of ammonia. Such solutions stain, but stain weakly and diffusely.

145. Carminic Acid occurs as a purple-brown mass, easily soluble both in water and in alcohol. It is (according to NIETZKI, *Chemie der organischen Farbstoffe*, Berlin, 1889, pp. 231—234) a weak (LIEBERMANN says a strong) dibasic acid, which forms soluble salts with the alkaline metals, insoluble violet-coloured ones with the earthy and heavy metals. Very little is known concerning the chemical nature of these salts.

The alumina salt (carminate of alumina) may be obtained by precipitating a solution of carminic acid or of carminate of ammonia by means of acetate of alumina. It has the remarkable property of being soluble not only in acids and acid salts, such as alum, but also in alkalies and alkaline salts, such as borax, provided that only water or weak alcohol be employed as the menstruum. It is also precipitated from the above-named solutions by chloride of aluminium, but only in part; whilst if alum be taken no precipitate is produced, the carminate of alumina remaining in solution. Hence the composition of the staining fluid given below under the name of **Carmalum.**

When chloride of aluminium is taken, a precipitate of carminate of alumina is formed, as stated above. But this precipitate will redissolve if more chloride of aluminium be cautiously added. This gives the staining fluid described in § 152, which may be convenient in cases in which it is not desirable to work with a fluid containing alum.

Both of these solutions stain in a violet tone, something like alum-carmine. A redder tone may be obtained by adding calcium chloride to the Carmalum solution. But this is not advisable, for calcium chloride added to Carmalum precipitates the solution with formation of gypsum. Of course, this does not occur with the aluminium chloride solution; but for other reasons the addition does not give satisfactory results with the chloride of aluminium solution mentioned above. But it does gives good results when combined with an *alcoholic* chloride of aluminium solution, and thus solves at once the problem of obtaining a *red* stain and an *alcoholic* staining fluid. This is described below under the name of **Paracarmine.**

If the foregoing explanations of the *rationale* of carmine

staining be compared with the remarks on the theory of staining with hæmatoxylin given below (§ 174) an interesting parallelism will be observed. In both processes, it is not the colouring matter alone which is active, but the colouring matter combined with *alumina*. The stain is always got with carminic acid + alumina, or with hæmateïn + alumina; other substances, such as lime, occasionally playing a part.

146. The foregoing considerations deal with the theory of staining with *Carmine;* we have now to consider the **Theory of Staining with Cochineal.** According to MAYER, whose earliest researches are confirmed by his latest (*Mitth. Zool. Stat. zu Neapel,* x, 3, 1892, p. 496), the active principle of extract or tincture of cochineal (as used in histology) is not free carminic acid, but carminic acid chemically combined with a base which is not lime, but some alkali. The pure aqueous extract contains only traces of lime, the alcoholic none at all. The watery extract made with *alum,* or cochineal-alum carmine (§ 154), owes its staining power to the formation of a carminate of alumina, the general properties of which have been discussed above when treating of the theory of carmine staining (§ 145). The tincture made with *pure alcohol,* on the other hand, contains only the above-mentioned carminate of some alkali. This carminate *alone* stains weakly and diffusely (like carminic acid alone). But if in the tissues treated with it it meet with lime salts, alumina or magnesia salts, or even metallic salts capable of combining with it and forming insoluble coloured precipitates in the tissues, then a strong and selective stain may result. As a matter of fact, the simple cochineal tincture of Mayer given in § 172 does give splendid results with certain objects (*i. e.* such as contain the salts in question). But it is unfortunately equally certain that such objects are rather rare than otherwise, and that with the majority of objects the stain is a very poor one.

But if the necessary salts be added to the tincture itself, then a solution ought to result containing the necessary elements for affording a strong and selective stain with all classes of objects. This proves to be the case; whence Mayer's new formula, § 173.

147. General Remarks.—What are the carmine stains useful for? Is it for staining fresh tissues? With the exception

of aceto-carmine, no. Is it for staining sections? Again, no; for, in nine cases out of ten, sections are better stained by some of the anilin stains than they can be in any carmine stain. Is it for staining entire objects?—for staining in the mass? Yes; for in many, if not in most cases, that can be done more satisfactorily by means of carmine than by means of any other known agent. So that until a coal-tar colour shall have been discovered that can beat alum-carmine and borax-carmine on their own ground, these must still hold their sway. As soon as that shall have been done—and it may be done any day—carmine stains will become as extinct as the dodo.

In view of the far greater simplicity and precision of the methods proposed by Mayer, it is probable that they, or at all events methods conforming to the principles above laid down, will gradually take the place of the old methods. Not, indeed, that some of the old stains will not hold their ground. Alum-carmine will remain a superb stain, and borax-carmine remains superior to paracarmine in the power and brilliancy of its stain; but the superior precision of Mayer's methods will doubtless be acknowledged. And it is perhaps not too much to hope that fewer formulæ of the old roundabout, happy-go-lucky sort, with commercial carmine or hæmatoxylin as the chief ingredient, will henceforth be published.

148. Pure Carminic Acid may be obtained from E. Merck of Darmstadt, or from Dr. G. Grübler (12, Bayersche Strasse, Leipzig). The price at present is from 2s. to 4s. per 10 grms.

149. Hints.—Overstains may in all cases be washed out with weak HCl (*e. g.* 0·1 per cent.). Henneguy (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 400) states that overstains may be completely removed by means of permanganate of potash. Sections thus treated may then be after-stained with anilin colours (see § 106). All carmine stains, with the exception of aceto-carmine, are permanent in balsam. Aqueous mounts should be acid (except for alum-carmine), and the best plan is to let the mounting medium contain 1 per cent. of formic or acetic acid. Formic acid is to be preferred.

Remember that none of the acid stains, except alum-carmine used as directed, § 155, nor any of Grenacher's fluids can be used with calcareous structures that it is wished to preserve.

Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with.

150. Classification of the Formulæ.—In the treatment of the for-

mulæ given in the following pages I have been guided by the considerations set out in § 147. The best stains for staining in the mass are fully treated, the old-fashioned solutions proposed for staining sections being thrown into the background. The formulæ set out below are arranged according to the nature of the menstruum. This gives us two great groups, aqueous carmine solutions and alcoholic carmine solutions. Taking first the group of aqueous solutions, I have arranged the formulæ comprised in it according to the reaction of the solutions.

The first subdivision comprises the acid solutions, *i. e.* those with free acid or acid salts. The second subdivision comprises the *so-called* neutral carmines and the alkaline solutions. These last are scantly treated, the very fact of a staining solution having an alkaline reaction and at the same time an aqueous menstruum implying that it must have a deleterious effect on tissues. Such stains should be reserved for tissues that have in the process of hardening been overcharged with chrome salts to such an extent as to no longer take up other stains, and for maceration preparations, for which they may sometimes be found useful. The alcoholic group is too small to require subdivision.

A. Aqueous Carmine Stains.

a. Acid.

151. Mayer's Carmalum (*Mitth. Zool. Stat. zu Neapel*, 10, 3, 1892, p. 489).—Carminic acid, 1 grm.; alum, 10 grms.; distilled water, 200 c.c. Dissolve with heat. Decant or filter. Add some antiseptic, either a few crystals of thymol, or 1 per cent. salicylic acid, or 5 per cent. salicylate of soda. The solution will then keep. A clearish red fluid with a violet tinge. It stains well in bulk even osmium objects. If washed out with distilled water only, the plasma will remain somewhat stained. If this be not desired, wash out carefully with alum solution, or, in difficult cases, with weak acid. The general effect is that of an alum-carmine stain. A notable difference between the two is that carmalum stains well in bulk, which alum-carmine is not very suitable for when used in the ordinary way; but see § 155.

A weaker solution may be made by taking from three to five times as much alum and five times as much water, and dissolving in the cold, which may be convenient. This is a very close equivalent of alum-carmine, giving, however, a somewhat redder stain. It should be noted that with either solution the objects to be stained should *not* have an *alkaline reaction*.

152. Mayer's Aqueous Carminate-of-Alumina-Solution (*Mitth.*

Zool. Stat. zu Neapel, 10, 3, 1892, p. 490).—Carminic acid, 1 grm.; chloride of aluminium, 3 grms.; water, 200 c.c. Add an antiseptic, as for carmalum.

Use as carmalum. The stain is of a blue-violet colour, very powerful, and elective. But it is not so pure a stain as that of carmalum, plasma being more strongly coloured. It is recommended only as a substitute for carmalum in cases in which the latter is counter-indicated on account of the presence of alum or the like.

153. Alum-Carmine (Grenacher's formula, *Arch. mik. Anat.*, xvi, 1879, p. 465).—An aqueous solution (of 1 to 5 per cent. strength, or any other strength that may be preferred) of common or ammonia alum is boiled for ten or twenty minutes with ½ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it dilute to the desired strength.) When cool filter.

This stain must be avoided in the case of calcareous structures that it is wished to preserve (but see § 155).

Tizzoni (*Bull. Sc. Med. Bologna*, 1884, p. 259) and Pisenti (*Gazz. degli Ospetali*, No. 24; *Zeit. f. wiss. Mik.*, ii, 1885, p. 378) add a small percentage of sulphate of sodium, with the object of enhancing the energy of the stain. It should not be forgotten that sodium sulphate is a substance that exercises a very peculiar action on nuclei (see Pfitzner, *Morph. Jahrb.*, xi, 1, 1885).

Grieb (*Mem. Soc. Ital. Sci.*, t. vi, No. 9, 1887; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 47) gives a modification of Grenacher's formula which does not appear to me rational.

It has already been sufficiently repeated that alum-carmine is one of the best stains to be found outside the coal-tar colours. It is particularly to be recommended to the beginner, as it is easy to work with; it is hardly possible to overstain with it (except muscle). Its chief defect is that it is not very penetrating, and therefore quite unsuitable for staining objects of considerable size in the mass. This defect may, however, be overcome by employing the method of Zoltán von Roboz (*infra*, § 155), or by employing the acid formula of Henneguy (§ 156), if it be not convenient to use Mayer's carmalum.

The stain is permanent in balsam, and in aqueous media if not acid.

154. Cochineal Alum-Carmine (Partsch, *Arch. f. mik. Anat.*, xiv, 1877, p. 180).—Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

Another method of preparation has been given by Czokor (*Arch. f. mik. Anat.*, xviii, 1880, p. 413). Mayer has carefully examined both, and finds that Partsch's is the more rational, the proportion of alum in it being exactly right, whilst in Czokor's it is insufficient. Partsch's fluid has also the advantage of keeping better.

The formula known as Klein's cochineal fluid (which appears to have been first published in the *Ann. and Mag. Nat. Hist.*, viii, 1881, p. 232) is identical with that of Czokor.

Both these solutions are to all intents and purposes "alum-carmines." They give a stain that is practically identical with that of alum-carmine made from carmine, with perhaps even more delicate differentiations (but that depends so much on the quality of the carmine, the quality of the cochineal, and the nature of the objects to be stained, that no absolute rule can be stated). On the whole it seems to be a mere matter of convenience whether the one or the other should be preferred. The cochineal fluids should be used in exactly the same way as the carmine fluid.

It has been lately recommended by Herrick (*Journ. Comp. Neur.*, Cincinnati, vol. i, 1891, p. 134) as a "vast improvement" to make the solution with sulphate of aluminium instead of alum (quoted from Mayer's paper, *Mitth. Zool. Stat. Neapel*, 10, 3, 1892, p. 496).

155. Alum-Carmine with Osmic Acid (Zoltán von Roboz, *in litt.*).—To 50 or 60 grms. of water is added alum-carmine until the mixture is of an almost red rose colour; about ten drops of a $\frac{2}{1000}$ solution of osmic acid are then added. (The mixture should have an appreciable smell of osmic acid.) The objects to be stained remain in the mixture for from twenty-four to forty-eight hours in the dark. It is hardly necessary to wash them, as the stain is perfectly precise without that. It is important to perform the staining in a well-closed vessel, in order to prevent the evaporation of the osmium.

I have used this stain with the most diverse objects, and *can most highly recommend it.* The result is a sharp nuclear

double stain (resting chromatin and nucleoli purple, kinetic chromatin red, the rest brown). I find it valuable for staining soft tissues in the mass. As the staining bath contains but very little alum, it may be used with calcareous structures, and is, indeed, for such objects as *Pluteus* about the best stain that I know of. It is difficult to understand why this admirable method has not been more used.

156. Acetic Acid Alum-Carmine (HENNEGUY, *Traité des Méth. techn.*, LEE et HENNEGUY, p. 88).—Excess of carmine is boiled in saturated solution of potash alum. After cooling add 10 per cent. of glacial acetic acid, and leave to settle for some days. The deposit of carmine and alum that forms during that time is removed by filtration.

For staining, enough of the solution is added to distilled water to give it a deep rose tint. Stain for twenty-four to forty-eight hours, and wash for an hour or two in distilled water. (It is important that the water should be distilled in order to avoid the formation of crystals.) Dehydrate with alcohol and mount in balsam. You can mount in glycerin, but the preparations do not keep so well as in balsam.

The advantage of this carmine is that it has great power of penetration, and stains deep-seated layers of tissue just as well as the superficial ones. The colour of the stain is a somewhat inelegant violet, but this can be changed to a warmer tone by treating the objects with dilute HCl, as for borax-carmine objects.

157. Alum-Carmine and Picric Acid.—Alum-carmine objects may be double-stained with picric acid. LEGAL (*Morph. Jahrb.*, viii, p. 353) combines the two stains by mixing ten vols. of alum-carmine with one of saturated picric acid solution.

GILSON's picro-alum-carmine (the stain mentioned by BOLSIUS in *La Cellule*, vii, 1, 1891, p. 6, kindly communicated by Prof. GILSON) is made by boiling 10 per cent. of cochineal for about twenty hours in saturated solution of alum (in a Liebig's refrigerator or some flow-back arrangement for preventing diminution of volume by evaporation), and adding 10 per cent. of saturated solution of picric acid.

158. Aceto-Carmine (Acetic Acid Carmine) (SCHNEIDER's formula, *Zool. Anzeig.*, No. 56, 1880, p. 254).—To boiling acetic

acid of 45 per cent. strength add carmine until no more will dissolve, and filter. (Forty-five per cent. acetic acid is the strength that dissolves the largest proportion of carmine.)

To use the solution you may either dilute it to 1 per cent. strength, and use the dilute solution for slow staining, which is the method to be preferred for making glycerin preparations; or a drop of the concentrated solution may be added to a fresh preparation under the cover-glass.

This is a very important reagent, which in certain cases renders services that no other reagent can render. If you use the concentrated solution it fixes and stains at the same time, and hence is most valuable for the study of fresh objects. It is very penetrating, a quality that enables it to be used where ordinary reagents would totally fail. The stain is a pure nuclear one. Unfortunately the preparations cannot be preserved, and for this and other reasons the stain is of very restricted application.

Zacharias (see *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 371) adds to this solution wood vinegar (*acetum pyrolignosum*) in the proportion of 1 drop to 10 c.c.

β. *So-called "Neutral," and Alkaline.*

159. As to Picro-carmine.—The term "picro-carmine" is commonly used to denote a whole tribe of solutions in which carmine, ammonia, and picric acid exist *uncombined* in haphazard proportions. Ranvier, to whom we owe the invention of picro-carmine, claims that when prepared by his process it results as a definite chemical substance, a double salt of picric and carminic acid and ammonia, or *picro-carminate of ammonia.* I am not prepared to say whether this is or is not the case, but I think it may be safely said that Ranvier's method of preparation is the only one yet published that will give with certainty a product having the desired qualities.

Prepared in this way picro-carmine gives very delicate differentiations, and has the great merit of being less hurtful to most tissues than other aqueous alkaline carmines. It is a single or a double stain according to the manner of using it. If the preparations be washed, after staining, with water, it is a *single* stain, the colour of the carmine alone appearing; if they be washed quickly in alcohol it is a *double* stain, the yellow coloration of the picric acid not being dissolved by the alcohol as it is by water. Of course the washing with alcohol must not be overdone, or the yellow coloration may be entirely removed. It should be understood that the chief value of picro-carmine does not lie in its capacity of affording a double stain. The double stain, if that is all that is wanted, can be just as well or better obtained by staining first with borax-carmine, or the like, and after-staining with picric acid. The essential point about picro-carmine is that it is a fairly *neutral*

fluid. Ranvier was, in fact, led to add picric acid to ammoniacal solution of carmine by the desire of neutralising the ammonia, that is all.

The good qualities (especially that of precision and delicacy of stain) above attributed to picro-carmine apply in their entirety only to Ranvier's picro-carmine or "picro-carminate of ammonia." The other pseudo-picro-carmines are in general so inferior as not to take rank as good stains at all. The reader is in consequence warned against an over-confident faith in them, and is especially warned against the so-called picro-carmine sold by the opticians. The true Ranvier's "picro-carminate of ammonia" can only be prepared *with certainty* by one process as yet known. This I proceed to give, and amongst the other formulæ set out one or two that *may* by chance give rise to the formation of a picro-carminate, and should at all events afford a useful staining solution (which the majority of these formulæ only do by chance).

For slow staining, dilute solutions may advantageously have 1 or 2 per cent. of chloral hydrate added to them.

Overstains may be washed out with hydrochloric acid, say 0·5 per cent., in water, alcohol, or glycerin.

Preparations should be mounted in balsam, or if in glycerin this should be acidulated with 1 per cent. of acetic or, better, formic acid.

160. Ranvier's Picro-carmine or Picro-carminate of Ammonia.—The method of preparation employed in the Laboratory of Histology of the Collège de France, kindly communicated to myself and Henneguy for our *Traité des Méth. techn.* (q. v., p. 451) by M. Vignal, one of the assistants there, is as follows:

Take—

Water	1000 parts.
Picric acid	20 „
Carmine	10 „
Ammonia	50 „

Put them into a stoppered bottle and leave them for two or three months in a warm place. Then put them into a large crystallising dish and let them putrefy. When the liquid has become reduced by evaporation to four fifths of its original volume, remove the crystals that have formed at the bottom, dry them, and dissolve them in a little warm water. Filter the solution, and examine it with the microscope to see whether the carmine is really dissolved. If not, add water and ammonia, and let the solution putrefy again; evaporate and examine as before. When you have got your carmine combined, evaporate the solution to dryness in a stove, and reduce the picro-carminate to powder.

For staining, dissolve 1 grm. of the powder in 100 grms. of water, and add a crystal of thymol to prevent the development of mould.

Ranvier's **Original Formula** (*Traité*, p. 100) was as follows:—To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one fifth the original volume in a drying oven; and separate by filtration the precipitate, poor in carmine, that forms in the liquid when cool. Evaporate the mother-liquor to dryness, and you will obtain the picro-carminate in the form of a crystalline powder of the colour

of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

161. Picro-carmine (*Weigert's formula, Virchow's Archiv*, Bd. lxxxiv, pp. 275, 315; *Zool. Jahrb.*, 1881, p. 40).—Two grms. of carmine are soaked for twenty-four hours (in a vessel protected from evaporation) in 4 grms. of ammonia; 200 grms. of concentrated solution of picric acid are then added, and the whole put away for twenty-four hours more. Small quantities of acetic acid are then added "until the first slight precipitate appears even after stirring." The whole is again put away for twenty-four hours more, when it will be found that there has formed a precipitate that can only partially be removed by filtration; ammonia is then added drop by drop at intervals of twenty-four hours, until the solution becomes clear. If the solution stains too yellow, acetic acid is added; if it overstains red, a little ammonia is again added. All badly staining samples of picro-carmine may be improved in the same way by addition of acetic acid or ammonia.

162. Other Formulæ for Picro-carmine.—Gage, *Am. M. Mic. Journ.*, i, 1880, p. 22; *Journ. Roy. Mic. Soc.*, vol. iii, p. 501 (very elaborate, and has not afforded me a soluble carmine). Fol, *Lehrb. d. vergl. mik. Anat.*, p. 195. Rutherford, *Pract. Hist.*, p. 173. Paul Mayer, *Mitth. Zool. Stat. Neapel*, ii, p. 20. Baber, *Mon. Micro. Journ.*, xii, p. 48. Pergens, Carnoy's *Biologie cellulaire*, p. 92. Hoyer, *Biol. Centralb.*, ii, 1882, p. 17. Bizzozero, *Zeit. f. wiss. Mik.*, 1885, p. 539. Klemensiewics, *Sitzb. Akad. Wiss. Wien*, lxxviii, 1878, iii, Juni; *Zeit. f. wiss. Mik.*, i, 1884, p. 501. Cuccati, *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 42.

For *Soda-Picro-carmine* see Löwenthal, *Anat. Anzeig.*, ii, 1887, No. 1, p. 22; Anon., *Journ. Roy. Mic. Soc.*, 1888, p. 518; Squire's *Methods and Formulæ, &c.*, 1892, p. 35.

163. Ammonia-Carmine.—In my opinion there is no valid excuse for using ammonia-carmine at all at the present day. The most that can be said of it is that it gives a good *general* stain of preparations of central nervous system that have been hardened in chromic acid or a chromic salt. But for central nervous system the stains described in Part II under the head of "Special Stains" are what is generally wanted.

If, however, such a stain be used, care should be taken to get rid of the free ammonia as completely as possible. This may be done by boiling until the excess of ammonia has evaporated. (So long as free ammonia is present *large* bubbles are formed in the fluid, and the latter shows a dark purple colour. When the free ammonia has evaporated *small* bubbles appear, and the solution takes a brighter red tint.)

One per cent. each of carmine and ammonia in distilled water is a good proportion.

But a safer mode of preparation is that of Ranvier, given below.

Staining is best done slowly, in dilute solutions, as many drops of the stain being added to distilled water as will give a liquid of a pale rose tint. Sections should remain in the liquid for twenty-four hours. In this way chromic objects stain well. For sections of encephalon the hardening *ought* to have been done in a chromic liquid, and all treatment with alcohol ought

to be carefully avoided until staining is accomplished; alcohol should not even be used for wetting the section knife.

Objects stained in strong solutions must be washed out in water, but not too long, as the stain will wash out too much in time. The colour may be "set," when required, by treatment with alcohol or a little acetic acid. Objects stained in the dilute sections recommended will not require washing out. Overstains may be reduced and made sharp by treatment with HCl alcohol, as for borax-carmine. The stain is permanent in balsam, or in aqueous media containing a little (about 1 per cent.) of acetic or (better) formic acid.

164. RANVIER'S "Neutral" Ammonia-Carmine (kindly communicated by Dr. MALASSEZ, see *Traité des Méthodes techniques*, &c., of Lee and Henneguy, p. 82).—Make a simple solution of carmine in water with a *slight* excess of ammonia, and expose it to the air in a deep crystallising dish until it is entirely dried up. It should be allowed to putrefy if possible. Dissolve the dry deposit in pure water, and filter.

165. Other Ammonia-Carmines.—BEALE, *How to Work*, &c., 4th ed., pp. 109, 304. BETZ, *Arch. mik. Anat.*, ix, 1873, p. 112; GIERKE, *Zeit. f. wiss. Mik.*, i, 1884, p. 76 (these two are methods for the preparation of putrefied carmine, and are highly recommendable, but excessively lengthy). HUXLEY and MARTIN, *Pract. Elemen. Biol.*, p. 268. FREY, *Le Microscope*, p. 167. HOYER, *Biol. Centralb.*, ii, 1882, p. 17. BÖHN (not "BOHM"), *Arch. Anat. u. Phys.* (*Anat. Abth.*), 1882, p. 4. HEIDENHAIN, *Arch. f. mik. Anat.*, vi, 1870, p. 402.

166. Lithium-Carmine (ORTH, *Berlin. klin. Wochenschr.*, 28, 1883, p. 421).—Two parts and a half of carmine are dissolved in ninety-seven parts and a half of saturated solution of carbonate of lithium.

The solution stains with equal readiness alcohol objects and chromic objects. The stain is diffuse, but becomes restricted to nuclei on treatment with hydrochloric acid (1 per cent. in 70 per cent. alcohol). The colour is permanent both in balsam and glycerin. The only advantage of this solution is the readiness with which it stains tissues that refuse to stain in any other medium.

HAUG (*Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 47, and viii, 1, 1891, p. 52) has three elaborate modifications that appear to me wild and uncalled for.

γ. *Other Aqueous Carmines (Acid and Alkaline).*

167. Schweigger-Seidel's Acid Carmine (RANVIER, *Traité*, p. 99). **Hamann's Acid Carmine** (*Intern. Mon. f. Anat. u. Hist.*, i, 5, 1884; *Zeit. f. wiss. Mik.*, ii, 1885, p. 87). **Neutral Borax-Carmine** (NIKIFOROW, *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 337). **Neutral Borax-Carmine** (GRENACHER, *Arch. f. mik. Anat.*, xvi, 1879, p. 466). HAUG's modifications of this, *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 151, and viii, 1, 1891, p. 52. **Woodward's Borax-Carmine** (see *Monthly Micr. Journ.*, vii, 1872, p. 38; *Am. Quart. Micr. Journ.*, i, 1879, p. 220; *Journ. Roy. Mic. Soc.*, ii, p. 613).

H. Gibbes' Borax-Carmine (see *Journ. Roy. Mic. Soc.*, iii, 1883, p. 390). **Delage's Osmium-Carmine** (*Arch. de Zool. exp. et gén.*, iv, sér. 2, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 240). **Rollet's Carminroth** (see *Zeit. f. wiss. Mik.*, i, p. 91). **Perl's Soluble Carmine** (see Frey, *Das Mikroskop.*, 7 *Auf.*, and *Zeit. f. wiss. Mik.*, i, p. 91). **Carminic Acid** (see Dimmock, *Amer. Natural.*, xviii, 1884, pp. 324–7; and *Journ. Roy. Mic. Soc.*, 1884, pp. 471–4). **Boric Acid Carmine** (Arcangeli, see *Proc.-verb. Soc. Toscana Sc. Nat.*, 1885, p. 283; and *Zeit. f. wiss. Mik.*, 1885, p. 377). **Boric Acid Alum-Carmine**, Arcangeli, *ibid.* **Salicylic Acid Alum-Carmine**, Arcangeli, *ibid.* **Salicylic Acid Carmine**, Arcangeli, *ibid.* **Picric Acid Carmine**, Arcangeli, *ibid.* **Picric Acid Carmine**, Minot (see Whitman's *Methods in Mic. Anat.*, p. 42). **Uranium-Carmine**, Gierke, *Zeit. f. wiss. Mik.*, i, 1884, p. 92; Schmaus, *ibid.*, viii, 2, 1891; cf. *Münchener med. Wochenschr.*, 1891, No. 8, p. 147. **Carbonate of Soda Carmine**, Cuccati, *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 50.

b. Alcoholic Carmine Stains.

168. Mayer's **Paracarmine (Alcoholic Carminate-of-Alumina-and-Lime Solution)** (*Mitth. Zool. Stat. zu Neapel*, x, 3, 1892, p. 491).—Carminic acid, 1 grm.; chloride of aluminium, 0·5 grm.; chloride of calcium, 4 grms.; 70 per cent. alcohol, 100 c.c. Dissolve cold or warm, allow to settle, and filter. A light red liquid, specially adapted for staining in bulk, and the nearest approach to a substitute for Grenacher's alcoholic borax-carmine that has yet been discovered.

Objects to be stained *should not have an alkaline reaction.* Wash out sections, or objects intended to be sectioned, with pure 70 per cent. alcohol. Objects intended to be mounted whole may be washed out with a weak solution of aluminium chloride in alcohol, or if this be not sufficient, with 5 per cent. common acetic acid (or 2·5 per cent. glacial acetic acid) in alcohol.

For staining bulky objects with large cavities, such as *Salpa*, the solution should be diluted (with alcohol); and as this may cause precipitates to form during the staining, especially if the objects are not very clean, it is advisable to *slightly acidify the dilute solutions.*

Paracarmine gives a nuclear stain of a red colour, though not so fiery red as that of borax-carmine. Its points of superiority over borax-carmine are that it is not alkaline, therefore less hurtful to tissues; that it is more highly

alcoholic, therefore more penetrating; that it has less tendency to form granular precipitates in the interior of objects, and that it keeps perfectly without precipitating.

169. Alcoholic Borax-Carmine (GRENACHER, *Arch. f. mik. Anat.*, xvi, 1879, p. 466, *et seq.*).—Take a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax); dilute it with about an equal volume of 70 per cent. alcohol, allow it to stand some time, and filter. Or the mixture of carmine and borax solution is *allowed to stand for two or three days* and occasionally stirred; the greater part of the carmine will dissolve. To the solution is added an equal bulk of 70 per cent. alcohol; the mixture is allowed to stand for a week, and then is filtered. If on keeping more carmine is deposited, it must be refiltered.

Preparations should remain in the stain until they are thoroughly penetrated (for days if necessary), and then be brought (*without first washing out*) into alcohol acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until they are thoroughly penetrated, and may then be washed or hardened in neutral alcohol. Four drops of HCl is generally enough. Three drops I find not quite sufficient. The stained objects should remain in the acidulated alcohol till they acquire a bright transparent look (three to six hours).

For delicate objects, and for very impermeable objects, it may be well to increase the proportion of alcohol in the stain; it may conveniently be raised to about 70 per cent. The washing out, or decoloration, will be enormously facilitated if picric acid be added to the acidulated alcohol, but in this case the proportion of HCl should be reduced. It should not exceed that of 1 drop of HCl to 100 c.c. of alcohol, and the decoloration should be carefully watched, as the stain may easily be entirely washed out in this mixture. For this reason the process is not to be recommended in general; I merely mention it because it is well that the student should be acquainted with the reaction.

This stain is probably by far the most popular of any for staining in the mass. It is easy to use, and gives a most splendid coloration. But it is not so penetrating as is commonly supposed, and has the defect of sometimes forming

precipitates in the cavities of bulky objects which cannot be removed by washing out. And it must be remembered that the fluid is alkaline, and therefore not suitable for delicate cytological work. I believe that some otherwise excellent cytological work has been vitiated by over-confidence in this reagent, and will have to be done over again.

170. Alcoholic Hydrochloric Acid Carmine.—In view of the above-mentioned defects of borax-carmine—defective penetration, and a tendency to form insoluble granular precipitates in the interior of objects—it is desirable to possess a powerful staining medium more highly alcoholised and of acid reaction. Hydrochloric acid carmine possesses these qualities, and may be useful in the case of objects for which Mayer's paracarmine may not suffice. It may, for instance, be frequently useful in work on Arthropoda, especially the marine forms.

GRENACHER'S receipt (*Arch. f. mik. Anat.*, xvi, 1879, p. 468) will be found extremely troublesome by those who are not expert at neutralising. The following method, due to PAUL MAYER (quoted from GARBINI'S *Manuale per la Technica moderna del Microscopio*, first ed., p. 46), is easy and gives excellent results. Take 100 grms. of alcohol (either absolute or of any weaker grade), 1 or 2 drops of HCl, and *an excess* of carmine, and boil until you get a clear solution, taking care that there remain *an excess of carmine.* This ought to give a nuclear stain, without the aid of HCl for washing out. If overstaining or diffusion should occur, wash out with alcohol, *very slightly acidulated* with HCl.

BRASS (*Zeit. f. wiss. Mik.*, ii, 1885, p. 303) takes 100 c.c. of 70 per cent. alcohol, 15 drops of HCl, and an excess of carmine. An old formula of PAUL MAYER'S (*M. T. Zool. Stat. Neapel*, iv, 1883, p. 521; *Journ. Roy. Mic. Soc.* [N. S.], iv, 1884, p. 317), which gives a more powerful stain than the preceding, is as follows :

Four grms. carmine are dissolved in 100 c.c. of 80 per cent. alcohol with the addition of 30 drops of concentrated pure hydrochloric acid, and heated for about half an hour in the water-bath ; the solution is filtered whilst still hot, and the superfluous acid is carefully removed by the addition of caustic ammonia, added until the carmine begins to be deposited. This solution stains very rapidly (embryos of lobsters are stained in about a minute) and intensely, though diffusely ; the preparations must be washed out with HCl alcohol if a nuclear stain is required.

The heating of alcohol of so high a grade as 80 per cent. being troublesome, not to say dangerous, the process may be modified by dissolving the carmine in 15 c.c. of water acidulated with the HCl, adding 95 c.c. of 85 per cent. alcohol, and then neutralising with ammonia.

If it be desired to dilute any of these solutions it should be done with alcohol, not water, and alcohol should be taken for washing out.

Other Alcoholic Carmine Stains.

171. Alcoholic Boric Acid Carmine (Francotte, *Bull. Soc. Belge Mic.*, 1886, p. 48).—Carmine, 0·4 grm.; boric acid, 5 grms.; water, 25 c.c.; 90 per cent. alcohol, 75 c.c. Boil and filter.

Dutilleul's Picro-borax-Carmine (*Bull. Sci. Dép. Nord*, xvi, 1885, p. 371).—This is quoted here as having the distinction of being the very worst stain I have ever tried.

Baumgarten makes a "borax-picro-carmine" by adding crystals of picric acid to Grenacher's solution until it assumes a blood-red colour (*Journ. Roy. Mic. Soc.*, 1888, p. 676; 1889, p. 149).

172. Alcoholic Cochineal (Mayer's **Old Formula**, *Mitth. Zool. Stat. Neap.*, ii, 1881, p. 14).—Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter, and the resulting clear, deep red solution is fit for staining.

The objects to be stained must previously be imbibed with alcohol of 70 per cent., and alcohol of the same strength must be used for washing out or for diluting the staining solution, as water, or alcohol of a different strength, gives rise to turbidity and precipitation of colouring matter (the fluid holding in solution matters that are only soluble in alcohol of exactly that degree of concentration). The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$ per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes, larger animals require hours or days. In the latter case large quantities of the solution must be employed. Very thin sections and delicate objects are best stained in a very dilute solution.

A nuclear stain, slightly affecting protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of certain salts in them. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue elements of the same preparation. Glands

or their secretion often stain grey-green. In embryos of *Lumbricus* Kleinenberg found the vessels to stain red, their contents of an intense blue.

Acids lighten the stain and make it yellowish red. Caustic alkalies turn it to a deep purple.

The best stains are obtained in the case of objects that have been prepared with chromic or picric acid combinations, or with absolute alcohol. Osmic acid preparations stain very weakly unless they have been previously *bleached* (§ 575). The acid must be carefully washed out before staining, or a diffuse stain will result. The stain is permanent in oil of cloves and balsam.

The object for which this stain was imagined is to obtain an *alcoholic* staining fluid whose high penetrating power allows it to be employed in the case of organisms, such as Arthropoda, whose chitinous investments are but very slightly permeable by aqueous solutions of carmine.

I have treated this stain at considerable length because I am convinced that it ought to be better known. It is very useful in many cases (Annelids, for instance), and indispensable for Arthropoda. On the other hand, it gives a very poor stain with unsuitable objects, and the majority of the objects with which the histologist has to do are in this case. For this reason Mayer has been led to devise the fluid described in the following paragraph, which, containing in itself the salts necessary for producing a rich and energetic selectivity of stain, gives good results with *all classes of objects*. He almost appears to intend it entirely to take the place of the old fluid. But I think the old fluid cannot yet be discarded. It has over the new fluid the (for some cases considerable) advantage of being more highly alcoholic; and it does not contain free acid, so that it can be used with calcareous structures which it is wished to preserve—which the new fluid cannot.

173. Mayer's **Alcoholic Cochineal, New Formula** (*Mitth. Zool. Stat. Neapel*, 10, 3, 1892, p. 498).—Cochineal, 5 grms.; chloride of calcium, 5 grms.; chloride of aluminium, 0·5 grm.; nitric acid of 1·20 sp. gr., 8 drops; 50 per cent. alcohol, 100 c.c. Powder the cochineal finely and rub up in a mortar with the salts, add the alcohol and acid, heat to boiling-point, leave to

cool, leave for some days standing with frequent agitation, filter.

Use as the old tincture, the objects being prepared and washed out with 50 per cent. alcohol. The stain is like that of paracarmine, but not quite so strong and not so sharp. Mayer only recommends it as a *succedaneum* of paracarmine. The object for which it was devised has been explained in the last paragraph, where also some limitations to its usefulness have been indicated.

For the chemical theory involved in the composition of these tinctures see also the last paragraph, and *supra*, § 146.

CHAPTER XI.

HÆMATEÏN (HÆMATOXYLIN) AND OTHER ORGANIC STAINS.

A. *Hæmatoxylin and Hæmateïn.*

174. Theory of Hæmatoxylin Staining.—It appears to be now thoroughly well established (see NIETZKI, *Chemie der organischen Färbstoffe*, Berlin, Springer, 1889, pp. 215—217) that the active colouring principle of hæmatoxylin dyes is *hæmateïn;* and further, that the hæmateïn of the usual histological staining solutions is a product of the oxidation of their contained hæmatoxylin by means of the air to which they are exposed (see MAYER, "*Ueber das Farben mit Hämatoxylin,*" in *Mitth. a. d. Zool. Station zu Neapel*, Bd. x, Heft 1, 1891, pp. 170—186; UNNA, "*Ueber die Reifung unserer Färbstoffe,*" in *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 483). This change is known as "ripening," and until it has taken place the solutions are not fit to use for staining.

Hitherto it has been the practice to rely (quite unconsciously so far as the chemical theory is concerned) on the spontaneous absorption by the solutions of oxygen from the air to effect this "ripening," but it has now been discovered (by both MAYER and UNNA independently) that nothing is easier than to bring about the reaction artificially; all that is necessary being, for instance, to add to a solution of hæmatoxylin containing alum a little neutralised solution of peroxide of hydrogen. The solution becomes almost instantaneously dark blue, "ripe," and fit for staining, thus definitively confirming the truth of the hypothesis.

Mayer goes further. A solution of pure uncombined hæmateïn would not afford a selective stain such as we require in histology; it would be at most a dye. The usual solutions (I am not here speaking of Weigert's or similar processes) all contain alum, and Mayer holds that the active agent in them is a compound of hæmateïn with alumina

("Hæmateïn-Thonerde"—Hæmateïn-alumina). He holds that this salt is precipitated in the tissues (chiefly in the nuclei) by certain organic and inorganic salts there present (for instance, phosphates); perhaps also by other organic bodies belonging to the tissues. Such salts are known to exist in living tissues. By the process of "fixing" they are fixed there,—either in the same chemical state, as when such an agent as alcohol is used for fixing; or as fresh compounds formed by the action of the fixing agent itself, as when such a fixing medium as sublimate is used.

The chief factor in the composition of these stains is, therefore—after the colouring substance itself,—*alumina*, to which, however, may be added some other bodies, such as lime, which may play a secondary rôle (as, for instance, chloride of calcium does in Kleinenberg's solution).

It follows that the first difficulty with which the worker with hæmatoxylin stains has to contend is that of getting his hæmatoxylin duly oxidised into hæmateïn, in order to the formation of the desired hæmateïn-alumina compound, or lake. If this be done by the hitherto customary process of leaving the solutions to "ripen" by the action of the air, a second difficulty arises; the oxidising process continuing, the solutions become "over-ripe," the hæmateïn, through further oxidation, passes over into colourless compounds, and the solutions begin to precipitate. They are therefore, in reality, a mixture in constantly varying proportions of "unripe," "ripe," and "over-ripe" constituents (the first and last being useless for staining purposes), and, in consequence, their staining power is very inconstant.

These difficulties are only very imperfectly got over by any of the very various methods that have been proposed until lately. Now, however, thanks to the important discoveries of Mayer and Unna, a great step in advance has been taken.

The great point in Mayer's work is that not hæmatoxylin, but *hæmateïn*, should be taken in the first instance for making the staining solutions. This at once relieves us from the tedious and uncertain process of "ripening" in the old way. We have a ripe solution to begin with. A discovery of Unna's, to be mentioned below, affords a means of preventing the "over-ripening" brought about by excessive oxidation.

In face of the very great advantages offered by the mode of preparation worked out by MAYER I have no hesitation in saying that the old methods ought to be entirely discarded, and Mayer's formulæ only, or at least only such formulæ as conform to the principles laid down by him, used instead. If this be done the methods of Unna will come to be of but secondary importance; but they may still be occasionally useful, and for that reason, as well as for their theoretical interest, I proceed to set them out in the following paragraphs. For MAYER'S hæmateïn methods see §§ 179 *et seq.*

175. UNNA'S **Ripening Method** (*Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 483).—It has been explained above that the spontaneous "ripening" of hæmatoxylin solutions is due to the absorption by them of oxygen from the air, and the consequent oxidation of their hæmatoxylin into hæmateïn. UNNA has found (as MAYER had found before him) that a freshly prepared light red alum solution of hæmatoxylin can be instantly ripened—that is, converted into a dark blue solution of hæmateïn of even much greater staining power than spontaneously ripened solutions—by the simple addition of a little neutralised peroxide of hydrogen. Such an **instantaneously ripe solution** may be conveniently prepared as follows:—Make a solution of crystallised hæmatoxylin in alcohol, and another solution of about five times as much alum in water, and mix the two solutions in a test-tube. Then pour a little commercial peroxide of hydrogen solution into a watch-glass, and add a crystal of soda. Remove the soda as soon as a blue litmus paper remains blue in the solution, pour the solution into the test-tube, and heat for a moment over a flame. The staining bath is now ripe, and must be used for staining *at once*, as precipitation of the hæmateïn begins to set in the moment the state of ripeness is attained.

176. UNNA'S **Half-ripe Constant Stock Solution** (*ibid.*).—It is inconvenient to have to make a fresh solution of hæmatoxylin every time it is desired to prepare a ripened solution for staining. Unna, therefore, sought for a means of preventing the continuance of spontaneous oxidation of hæmatoxylin solutions, which, as explained above, leads in course of time to *over-ripeness*. The spontaneous oxidation being arrested at any desired stage, a *constant half-ripe* solution is obtained,

which may be at any moment converted into a totally ripe one by the process given in the last §.

This result maybe attained by simply adding a *reducing agent* to the solution. Various reducing agents are available for this purpose (see the original paper) ; the most convenient method is the simple addition of a little sulphur. The following formula is recommended :

Hæmatoxylin	1
Alum	10
Alcohol	100
Water	200
Sublimed sulphur	2

If the sulphur be added to the hæmatoxylin solution only when the latter has become somewhat strongly blue, *i. e.* after two or three days' time, the stage of oxidation attained by the solution will be fixed by the sulphur. The solution in this state may be used for staining. It will not give so energetic a stain as the solutions totally ripened with peroxide of hydrogen, and the stain will be somewhat more diffuse, but washing out with acids will not be necessary. If an energetic and more purely nuclear stain be desired, the solution should be treated with neutralised peroxide of hydrogen, as described in the last § ; sections should be stained in it at once, and quickly brought into glacial acetic acid (where they should only remain for a moment), or into HCl alcohol, for washing out.

177. General Remarks.—We have the coal-tar colours for staining sections, and we have carmine and cochineal for staining in the mass. What, then, do we want hæmatoxylin for? The answer is that we sometimes want it for staining in the mass on account of the faculty it has of readily staining tissues that have been treated with chromic and osmic mixtures. This it does in general better than any carmine or cochineal. We want it also for some special purposes, such as staining the *Nebenkern* and achromatic figure of nuclei, and for nerve researches and other special histological objects.

None of the old solutions of hæmatoxylin are perfectly stable ; only one or two are fairly so.

It has been explained above that when freshly prepared they stain badly and diffusely; they ought either to be "ripened" by Unna's method(§ 175) or to be allowed to "ripen" spontaneously before use. This takes, according to the nature of the solution, a few hours, or days, or months. On the other hand, kept solutions easily go bad by precipitating, or becoming acid, or becoming mouldy.

Most of the solutions, when in good staining order, have a great tendency to over-stain. Over-stains may be corrected by washing out with weak acids (*e. g.* 0·1 to 0·2 or even 0·5 per cent. of hydrochloric acid), but this is not favorable to the permanence of the stain. If acids be used, it is well to re-neutralise afterwards with ammonia or bicarbonate of soda (0·1 per cent.).

A better plan is, perhaps, to wash out with alum solution; but this frequently requires great patience.

The stain is fairly permanent in balsam, but is sure to fade a little, and may fade a great deal. If acids have been used after staining great care should be taken to wash them out thoroughly before mounting. In aqueous media the stain cannot be relied on to keep (this refers to the old solutions: Mayer finds that his hæmateïn preparations have kept well both in aqueous media and, with certain precautions, in balsam).

178. Hæmatoxylin stains in **different tones of blue or of red,** according to the composition of the staining solution.

According to WATNEY (see *Phil. Trans.*, 1882, p. 1075; KRAUSE, *Intern. Zeit. f. Anat. u. Hist.*, i, p. 154; and M. FLESCH, *Zeit. f. wiss. Mik.*, 1885, p. 358, from whom I quote) the colour is an intense blue if the solution has been made *with freshly prepared alum*, whilst a red tone is obtained if the solution has been made with old alum. The reason of this is that alum that has been long kept almost always contains free acid. The red solutions exhibit a great affinity for connective tissue, and for the granules of "plasma-cells;" whilst the blue solutions show a special affinity for mucin and chromatin. MAYER (*Mitth. Zool. Stat. Neapel*, 10, i, 1891, p. 177) has a different explanation of this reaction. He finds that solutions containing only hæmateïn and aluminium chloride stain mucin with special energy; whilst solutions which also contain from 10 to 20 per cent. of magnesium chloride, nitrate of ammonia, chloride of lithium or of calcium, of sodium, or of ammonium, do not do so, but are pure nuclear stains.

It has been discovered by LANGHANS (see MAX FLESCH, *loc. cit.*) that it is possible to obtain these two elective reactions with one and the same solution. All that is necessary is to stain with the solution of Delafield, mount the

preparations in balsam, and expose them for some time to the light. The reaction is not obtained with glycerin mounts.

In order to *get a blue stain* in preparations that have come out red through the acidity of the staining bath, it is usual to treat them with weak ammonia, in the belief that the blue colour is restored by neutralisation of the acid that is the cause of the redness. According to MAYER, the ammonia acts not by neutralising the acid, but by precipitating the alumina, which carries down the hæmateïn with it (if no alumina were present the colour would be purple, not blue). The *same result* can sometimes be obtained by merely washing out with common tap-water, which is usually sufficiently alkaline, and can be obtained with certainty by treatment with bicarbonate of soda or acetate of soda or potash. And this is the preferable course, as ammonia is certainly a dangerous thing to treat delicate tissues with. Of course this is a different question from that of neutralising with an alkali tissues that have been treated with an acid to correct over-staining. Here the neutralisation may be indicated in the interest of the *preservation* of the stain, or of the tissues themselves.

179. Concerning Hæmateïn.—The following is taken from the two papers of Mayer quoted above, §§ 144, 174. Hæmateïn occurs as a dark green metallic mass, red by transmitted light, which may be rubbed up into a violet powder or small red crystals. It is entirely, though with difficulty, soluble in distilled water and in alcohol, giving a yellowish-brown solution, which remains clear on addition of acetic acid. Alkalies dissolve it with a blue-violet tint.

It is now found in commerce ; but Mayer has hitherto only been able to procure it in a pure state from GEIGY AND CO., in Bâle. But there is also found in commerce an ammonia-compound of hæmateïn—*Hæmateïn-Ammoniak,* also known in commerce as *Hæmateïnum crystallisatum ;* this may be obtained in a sufficiently pure state from E. MERCK, of Darmstadt.

This is somewhat more easily soluble in both water and alcohol than hæmateïn is, and does quite as well for staining purposes. The histologist can easily prepare it for himself as follows :

Dissolve 1 grm. of hæmatoxylin with the aid of heat in 20

c.c. of distilled water, filter if necessary, add 1 c.c. of caustic ammonia (of 0·875 sp. gr.), and bring the purple liquid into a capsule of such dimensions that its bottom be not covered to a depth of more than half a centimetre. Let the liquid evaporate at the ordinary temperature and protected from dust. The dry product will be hæmateïn-ammonia, about equal in weight to the hæmatoxylin taken in the first instance. The evaporation should not be hastened by heat, as this may give rise to the formation of substances that are insoluble in alcohol. The preparation should not be touched, until it is dry, with any other instruments than such as are made of glass, porcelain, or platinum.

B. *Alumina Hæmateïn Stains.*

180. MAYER'S **Hæmalum** (**Hæmatein-alum Solution, Hæmalaun**; MAYER, *Mitth. a. d. Zool. Stat. zu Neapel*, 10, 1, 1891, p. 172; see also *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 338, a very exact report, but somewhat short considering the importance of Mayer's paper).—One grm. of the colouring matter (either hæmateïn or the ammonia salt) dissolved with heat in 50 c.c. of 90 per cent. alcohol, and added to a solution of 50 gr. of alum in a litre of distilled water. Allow the mixture to cool and settle, filter if necessary, and add a crystal of thymol to preserve from mould.

A dark liquid of about the tint of Grenacher's borax-carmine. It answers to Böhmer's hæmatoxylin,* stains at least as well, *either at first, for it is ripe from the beginning, or later.* It may be used either in the concentrated form or diluted. Concentrated, it stains almost instantaneously (sections have been stained by merely pouring the liquid over them). Diluted twentyfold with distilled water it will still stain through the tentacles of a *Tubularia* in an hour. (Spring water or tap water containing lime must not be used for diluting; perhaps weak solution of alum in distilled water is the best means of all.) After staining, sections may be washed out either with distilled or common water. The solution is *admirable for staining in bulk.* Large objects will,

* As with Böhmer's formula, it is not necesssary to conform exactly to the proportions given, and a rough and ready hæmalum solution may be at any time extemporised by adding a few drops of alcoholic solution of hæmateïn to an alum solution of any desired strength.

however, require twenty-four hours' staining, and should be washed out for the same time (this should be done with 1 per cent. alum-solution if a sharp nuclear stain be desired).

Mayer's preparations have kept well in glycerin (care being taken not to have it acid), also in balsam. It is to be noted that if oil of bergamot be used for clearing, it must be thoroughly removed by means of oil of turpentine before mounting; and that oil of cloves is dangerous.

Hæmalum may be mixed with alum-carmine, Säurefuchsin, or the like, to make a double-staining mixture; but it seems preferable to use the solutions in succession.

181. MAYER'S **Acid Hæmalum** (*ibid.*, p. 174, note).—This is hæmalum with 2 per cent. glacial acetic acid (or 4 per cent. common acetic acid). To be used as the last, washing out with ordinary water in order to obtain a blue-violet tint of stain. It has the advantage of being a perhaps even more precise nuclear stain than the last formula; and further, the solution does not precipitate through decomposition caused by the ammonia of the air and the alkali of the glass of bottles, which the simple hæmalum solution does to a certain extent (though this does not affect its staining power).

182. MAYER'S **Hæmacalcium** (*ibid.*, p. 182).—Hæmateïn (or its ammonia-salt), 1 grm.; chloride of aluminium, 1 grm.; chloride of calcium, 50 grms.; glacial acetic acid, 10 c.c. (or common acetic acid, 20 c.c.); 70 per cent. alcohol, 600 c.c. Rub up finely together the first two ingredients, add the acid and alcohol, dissolve either cold or with heat; lastly add the chloride of calcium.

A reddish-violet liquid, imagined as a substitute for KLEINENBERG'S solution, in view of the many disadvantages of the latter (see below, § 184). To be used in the main as Kleinenberg's. If the objects stain in too red a tone they should be treated with a solution (of about 2 per cent.) of chloride of aluminium in alcohol, or with a 0·5 to 1 per cent. solution of acetate of soda or potash in absolute alcohol, or with bicarbonate of soda (see above, § 178).

The solution is not perfectly stable, but in course of time (*Mitth. a. d. Zool. Stat. Neapel*, 10, 3, 1892, p. 499) turns blue and precipitates. To avoid this the mixture should be

made up in two separate bottles, each containing half of the alcohol and of the acid, and one containing besides all the calcium chloride, the other all the hæmateïn and all the aluminium chloride; equal quantities being taken from each when required for staining.

With certain objects this solution does not penetrate well, the stain being confined to their superficial parts. This may be remedied by acidifying the solution, or, which is better, by leaving the objects for some time before staining in acid alcohol. Anyway, objects ought NOT to have an alkaline reaction. If these precautions be taken, it will not be necessary to use acid for washing out. For some objects also (*e. g.* Hydroida) the penetrating effect is enhanced by diluting the solution with one third vol. of glycerin, or by increasing the proportion of aluminium chloride up to about eight times that of the hæmateïn.

This solution is not recommended as giving as good results as hæmalum,—as a stain it is distinctly inferior; and Mayer is of opinion that no alcoholic hæmateïn solution can be made to give as precise a stain as the aqueous solutions, for he holds that the watery nature of the menstruum is of itself of a beneficial effect so far as regards the *quality* of the stain. He recommends it merely as a substitute for Kleinenberg's (in cases in which an alcoholic hæmateïn stain seems indicated), as being convenient, easy to prepare, and constant in its effects, none of which qualities belong to Kleinenberg's formula (see below, § 184).

183. MAYER'S **Nitrate of Ammonia Hæmateïn Stain** (*Mitth. a. d. Zool. Stat. zu Neapel*, 10, 1, 1891, p. 183, note); HENKING (*Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 340) has proposed for this the name of **Hæmammon**).—Hæmalum, 10 c.c.; 70 per cent. alcohol, 10 c.c.; nitrate of ammonia, 5 grms. Dissolve; let the solution stand overnight for the excess of alum to crystallise out, and filter.

Very good for small objects (Mayer has not tried large ones), but does not give so good a stain as hæmalum; so that, of course, its only *raison d'être* is that, like hæmacalcium, it is an alcoholic solution.

184. Kleinenberg's Hæmatoxylin (*Quart. Journ. Mic. Sci.*, lxxiv, 1879, p. 208).—Prepare a saturated solution of calcium chloride in 70 per

cent. alcohol, with the addition of a little alum; after having filtered, mix a volume of this with from 6 to 8 volumes of 70 per cent. alcohol. At the time of using the liquid pour into it as many drops of a concentrated solution of hæmatoxylin in absolute alcohol as are sufficient to give the required colour to the preparation, of greater or less intensity according to desire.

Mayer (*Mitth. a. d. Zool. Stat. zu Neapel*, 10, 1, 1891, p. 174) has an elaborate criticism on this formula, from which I extract the following:— The object of the chloride of calcium is explained by Kleinenberg to be the setting up of diffusion currents between the alcohol in the tisssues and the external staining medium, so as to facilitate the penetration of the latter. Mayer himself does not admit this; he holds that it acts chemically, and that its effect is to make the stain more precise. I gather (though this is not quite clear) that he holds that it acts in this way *both* as a calcium salt, and also, after double decomposition, with the alum, as aluminium chloride, the presence of this salt being the most important factor in the production of the stain. Now Kleinenberg's formula does not efficiently provide for this double decomposition and formation of aluminium chloride. For cold 70 per cent. alcohol dissolves hardly any alum,* so that if the alcohol preserved its strength when the calcium chloride is dissolved in it, it would dissolve hardly any alum, and consequently hardly any aluminium chloride could be formed. But as calcium chloride contains about 50 per cent. of water of crystallisation the alcohol becomes gradually weaker, and in consequence dissolves more chloride of calcium, until at last you get a dense liquid containing 70 per cent. of the salt. If now alum be added to this, you get a precipitate of gypsum which envelops the crystals of alum, and protects them so that they no longer dissolve. And at the end the liquid contains only a mere trace of aluminium chloride. If you take hot alcohol you will dissolve more alum and obtain more aluminium chloride, but you will also decompose much of your alum by the heat, and thus get a strongly *acid* liquid, not fit for staining (the sulphuric acid set free from the alum combines with the calcium chloride, forming gypsum and setting free *hydrochloric* acid).

A further point is the vagueness of Kleinenberg's formula as regards the amount of hæmatoxylin to be added to make the staining bath. If too much be taken, as often happens, precipitates will be formed on the surface of the objects, hindering the penetration of the stain, and obliging you to wash out with acidified alcohol to remove them.

It was suggested long ago by Mayer (*Mitth. a. d. Zool. Stat. zu Neapel*, 2, 1880, p. 13) that it might be more convenient to avoid the douple decomposition by taking aluminium chloride in the first instance instead of alum. And, accordingly, two formulæ founded on this principle have been given by Dippel (in his *Handbuch der Mikroskopie*). But these formulæ both omit the calcium chloride, and give in consequence a blue-violet liquid which furnishes a diffuse stain (as is the case with all solutions containing a relatively large proportion of aluminium salt [chloride or nitrate] with relatively little hæmateïn). This makes it necessary to over-stain strongly and wash out

* Boiling 70 per cent. alcohol does not dissolve 0·5 per cent. of alum, almost all of which falls out on cooling.

with acid. A good staining solution should not be blue-violet; if it be, it should be corrected by cautious addition of acid. But it is better to avoid having an excess of aluminium salt.

Mayer then gives some hints concerning the ingredients of staining solutions such as we are here considering. *Aluminium chloride* should not be damp. It should have a strongly acid reaction. The *alcohol* and the *vessels* used should *not* have an alkaline reaction. The test for this is that a weak solution of hæmateïn in the alcohol should not turn purple; or that a solution of 1 part each of hæmateïn and aluminium chloride in 100 parts of alcohol being added to the alcohol, in the ratio of 1 : 100, should not precipitate in the course of twenty-four hours. The *calcium chloride* should have a neutral or feebly alkaline reaction, and should not be damp.

In view of the above criticism, the correctness of which, in the main at least, will be acknowledged by all who have worked with Kleinenberg's solution, I think it is quite time that it should be discarded, and Mayer's hæmacalcium used instead in all cases in which a powerful, alcoholic hæmateïn stain is indicated (*e. g.* for staining in bulk large impermeable objects that have been fixed in osmic or chromic mixtures).

If, however, it be used, the following points should be observed :—The solution should be allowed to ripen before using (Kleinenberg's practice was the contrary of this, and, I conceive, erroneous). Small objects are best stained slowly with a very dilute solution. If it be required to dilute a solution already prepared for staining, this should not be done with alcohol, which may easily cause precipitates to form on the tissues, but with the above-described solution of alum in calcium chloride solution. Over-stains should be washed out with acidulated alcohol. Either oxalic or (½ per cent.) hydrochloric acid may be used, and the specimens allowed to remain in them until they begin to acquire a reddish hue. The acid is then removed by pure alcohol, which restores the pure blue of the stain. It must be very thoroughly removed in order to ensure the permanence of the stain.

For large or impermeable objects immersion for days in a very strong solution may be necessary for staining. Osmium and chromic acid objects stain sufficiently.

185. Delafield's Hæmatoxylin (*Zeit. f. wiss. Mik.*, ii, 1885, p. 288).—The history of this formula is as follows :—It had long been in use in the Institute of Pathology at Heidelberg, when it was communicated by Pfitzner to Flemming, who published it and particularly recommended it in his *Zellstz.*, &c., p. 388, 1882. Flemming then attributed the formula to Grenacher, and in consequence the stain went for years by the name of "Grenacher's hæmatoxylin." Later on Flemming discovered that this attribution was erroneous, and attributed the formula to Prudden; and in consequence it was thenceforth known for some time as "Prudden's hæmatoxylin." In 1885 matters were set right by Prudden's explaining that the stain was the invention of Delafield, and publishing the correct formula here quoted (in the formula as published by Flemming the proportions are somewhat different).

To 400 c.c. of saturated solution of ammonia-alum* add

* Ammonia-alum dissolves in about 11 parts of water.

4 grms. of hæmatox. crist. dissolved in 25 c.c. of strong alcohol. Leave it exposed to the light and air in an unstoppered bottle for three or four days. Filter, and add 100 c.c. of glycerin and 100 c.c. of methylic alcohol (CH_4O). Allow the solution to stand until the colour is sufficiently dark, then filter and keep in a tightly stoppered bottle.

This solution keeps well,—it may be said to keep for years. It is well to allow it to ripen for at least two months before using it.

This famous solution is certainly one of the best hæmatoxylin stains yet published. It is extremely powerful, and when properly used very precise. For staining, enough of the solution should be added to pure water to make a very dilute stain; and even then care should be taken not to leave objects too long in the fluid.

Bütschli (*Unters. üb. mikroscopische Schäume u. das Protoplasma,* &c., 1892; *Zeit. f. wiss. Mik.,* ix, 2, 1892, p. 197) recommends, under the name of "acid hæmatoxylin," solution of Delafield very strongly diluted, and with enough acetic acid added to it to give it a decidedly red tint. This gives a sharper and more differentiated nuclear stain than the usual solution.

186. Böhmer's Hæmatoxylin (*Arch. f. mik. Anat.,* iv, 1868, p. 345; *Aerzt. Intelligenzbl., Baiern,* 1865, p. 382).—Make (A) a solution of hæmatox. crist. 1 part, alcohol (absolute) 12 parts, and (B) alum 1 part, water 240. For staining, add two or three drops of A to a watch-glassful of B.

The staining solution ought not to be made up at the moment of using, but should be made up beforehand and allowed to ripen for some days (Mayer says at least two months). The alcoholic solution of hæmatoxylin may be kept in stock; it becomes brown, but does not lose its properties.

Washing out may be done with a 0·5 per cent. solution of alum in water, or with acids if desired.

This formula gives perhaps a finer stain than Delafield's, but, on account of the uncertainty of the ripening process, is not so certain in its results.

187. Ranvier's Hæmatoxylin (*Comptes rend. Ac. Sc.,* 2 sem., t. xcv, p. 1375).—If solutions prepared according to the formula of Böhmer be kept for some weeks they will be found to furnish an abundant precipitate.

Ranvier re-dissolves this precipitate in 1 per cent. solution of alum, and employs the solution for staining sections of epidermis that have been hardened in bichromate of potash. The sections should remain twenty-four hours in the liquid. Nuclei are then found stained light violet, the granules of eleïdin dark violet. CONTEJEAN (*Bull. Soc. Philomath. Paris*, 8, 3, 1891, p. 117) has given a receipt for preparing this product by precipitating solution of Böhmer with baryta water. (Cf. Mayer, in *Mitth. a. d. Zool. Stat. zu Neapel*, 10, 3, 1892, p. 500.) The stain is a very fine one ; but critically examined, these methods appear to be only roundabout methods of getting a solution of hæmateïn in alum.

188. Ehrlich's Acid Hæmatoxylin (*Zeit. f. wiss. Mik.*, 1886, p. 150).—The ordinary (alum) hæmatoxylin staining solutions easily decompose, giving rise to a blue precipitate which is formed by the splitting up of the alum into free sulphuric acid and a basic, lake-forming compound of alumina. By adding to a solution an appropriate acid this decomposition may be prevented. The end may be attained by acetic acid. Take—

Water	100 c.c.
Absolute alcohol . . .	100 „
Glycerin	100 „
Glacial acetic acid . . .	10 „
Hæmatoxylin	2 grammes.
Alum in excess.	

Let the mixture ripen in the light (with occasional admission of air) until it acquires a dark red colour. It will then keep, with a perfectly constant staining power, for years, if kept in a well-stoppered bottle. Sections are stained in a few minutes. The stain is also very appropriate for staining in bulk, as over-staining does not occur.

In order to get a blue stain with this acid solution, the stained objects should be treated as directed *supra*, § 178, but common tap-water may suffice.

For double-staining, either acid colouring matters ("Färbsäuren"), such as eosin, or "basic colouring matters" ("Färbbasen"), may be added to the solution.

This is one of the most important of the alum hæmatoxylins.

188a. Ammoniated Hæmatoxylin (SQUIRE, *Methods and Formulæ, &c.*, p. 24).—"Hæmatoxylin, 2 grms.; ammonium carbonate, 0·4 grm.; proof spirit, 40 c.c. Dissolve the ammonium carbonate and the hæmatoxylin in the proof spirit

and expose to the air in a shallow dish for twenty-four hours; make up the volume to 40 c.c. with proof spirit (warming if necessary to dissolve any separated crystals), and add ammonia alum, 2 grms., dissolved in distilled water, 80 c.c.; glycerin, 100 c.c.; rectified spirit, 80 c.c.; glacial acetic acid, 10 c.c. This solution requires no 'ripening,' and is ready to be diluted for use (1—10)."

This hæmatoxylin is the one most recommended by Squire after extensive experimentation. I regret that up to the present I have not had time to make a trial of it, but have no doubt that it is excellent. Critically examined it is seen to correspond to Mayer's acid hæmalum, with the addition of alcohol and glycerin. I apprehend that the formula was arrived at without knowledge of Mayer's work on the subject, in which case the agreement between the two experimenters is very satisfactory. I am doubtful as to the advisability of the addition of alcohol and glycerin to alum-hæmateïn solutions. I suppose they help to make them keep, but do they not impair the quality of the stain?

189. Renaut's Glycerin Hæmatoxylin ("**Glycérine Hématoxylique**;" *Arch. de Physiol.*, 1881, p. 640).—Make a saturated solution of alum in strong glycerin. Add drop by drop about a quarter of a volume of concentrated solution of hæmatoxylin in alcohol. (If you add an excess of hæmatoxylin the liquid will become turbid, and you must then add more solution of alum in glycerin until the turbidity disappears.) Filter, and leave the solution exposed to the light and air for some weeks, until it can be perceived by the smell that it no longer contains any alcohol. Filter.

Sections are stained in this solution in a few minutes. But it can be used in another way, which is an original and really valuable method. If sections or other objects be mounted in a drop of the solution, they will, after a few weeks, be found stained and the glycerin decoloured. Such objects keep the stain for years.

190. Iodine Hæmatoxylin (Cuccati, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 55).—Dissolve 25 grammes of chemically pure potassic iodide in 25 c.c. of distilled water. Pour this solution gradually and with constant agitation into 75 c.c. of absolute alcohol

contained in a stoppered bottle. Close the bottle thoroughly. Rub up in a mortar 75 cg. of crystallised hæmatoxylin with 6 grammes of chemically pure roche alum,* and add 3 c.c. of the iodine solution. Keep the mixture agitated, and add gradually the rest of the iodine solution; then replace the whole in a well-stoppered bottle. Agitate for some time, in order to get the alum to dissolve, and let stand for ten to fifteen hours. Then shake well and filter, taking the usual precautions against evaporation of the alcohol, and preserve in a well-stoppered bottle.

Objects should be left in the liquid for ten hours, then well washed in water and mounted in glycerin, or washed in alcohol and mounted in balsam.

The solution is stated to be perfectly stable and to give a pure chromatin stain, and to be well adapted for staining in the mass, as it never over-stains.

191. Iodine Hæmatoxylin (Sanfelice, *Journ. de Microgr.*, xiii, 1889, p. 335; *Journ. Roy. Mic. Soc.*, 1889, p. 837).—Dissolve 0·70 grm. hæmatoxylin in 20 grms. absolute alcohol, and 0·20 grm. alum in 60 c.c. distilled water. Add the first solution, drop by drop, to the second. Expose the mixture to the light for three or four days, add ten to fifteen drops of tincture of iodine, agitate, and allow to stand for some days. Stain for twelve to twenty-four hours, and wash out for the same time in 90 per cent. alcohol, acidulated with acetic acid. The fluid keeps well.

Mayer (*Mitth. Zool. Stat. Neapel*, 10, 1, 1891, pp. 178, 182) remarks on this that it is essentially a Böhmer's solution with somewhat more alcohol, and stains well when ripe, the function of the iodine being to prevent the formation of precipitates of colouring matter on the surface of the objects, and not to directly increase the penetrating power of the stain.

192. Other Aqueous Alum-Hæmatoxylins.—Arnold, see *Quart. Journ. Mic. Sci.*, 1878, p. 86. Mitchell, see *Journ. Roy. Mic. Soc.*, 1884,

* Roche alum, or Roman alum (*allume di rocca, alun de roche, alumen rubrum verum*, and other synonyms), is an alum originally imported from Cività Vecchia, and much esteemed by dyers from being nearly free from iron-alum. That now sold for it in England is ordinary alum coloured with Venetian red, Armenian bole, or rose-pink (*alumen rubrum spurium*). See Cooley's *Cyclopædia of Pract. Receipts*, s. v. "Alum, Roman."

p. 811—a complicated method of treating logwood. HICKSON, see *Quart. Journ. Mic. Sci.*, 1885, p. 244—a still more complicated method. COOK, see *Journ. of Anat. and Phys.*, 1879, p. 140—a sulphate of copper solution. HAUG, see *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 51—an acetate of alumina solution.

c. *Other Hæmateïn Stains.*

193. Heidenhain's Hæmatoxylin (*Arch. f. mik. Anat.*, 1884, p. 468, and 1886, p. 383).—Stain for twelve to twenty-four hours in a $\frac{1}{3}$ per cent. solution of hæmatoxylin in pure water (distilled water only should be used). Soak the objects for the same length of time in a 0·5 per cent. solution of neutral chromate of potash. Wash out the excess of chromate with water, and treat further as desired.

The above is a slightly modified form of the original process, in which staining was done in a stronger hæmatoxylin solution (0·5 to 1 per cent.), and bichromate was used for washing out instead of neutral chromate. The more recent process gives a sharper chromatin stain.

The stain succeeds best with alcohol or picric acid objects, but it will succeed with chromic objects if they have been very well washed.

Objects that have been fixed in corrosive sublimate ought to be very carefully washed out with water (many hours in running water), as neutral hæmatoxylin forms a black precipitate with the excess of sublimate that remains after washing out with alcohol (see TORNIER, in *Arch. f. mik. Anat.*, 1886, p. 181).

The stain is black to grey (hæmatoxylin forming with chromic salts a black compound). It is a sharp stain, remarkably rich in detail.

The process is one well adapted to staining in the mass. Perhaps its greatest advantage lies in the fact that you can decolour the objects to any extent by prolonging the washing in the chromate.

The method may be varied by washing out after staining with alum solution (1 per cent.) instead of a chromate. In this case the stain will be blue.

194. Apáthy's Modification of Heidenhain's Process (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 47).—This is an *alcoholic* method.

Stain in a 1 per cent. solution of hæmatoxylin in 70 or 80 per cent. alcohol. Wash out (for "thin" sections, *i. e.* sections of 10 to 15 μ, half the time of staining—for "thicker" sections of 25 to 40 μ twice the time of staining) in 1 per cent. solution of bichromate of potash in 70 to 80 per cent. alcohol.

The bichromate solution is conveniently prepared by mixing one part of a 5 per cent. aqueous solution with about four parts of 80 to 90 per cent. alcohol. The mixture should be made immediately before using, and should be kept from the light (light precipitates it) during the process of decoloration, and should also be changed for fresh several times during the process. After the differentiation of the colour has been accomplished, the objects should be thoroughly washed (still in the dark) in several changes of 70 per cent. alcohol.

Preparations made in this manner are more transparent and better preserved than those made by Heidenhain's process.

For staining celloidin series of sections, Apáthy also (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 170) recommends the following procedure :—Stain in the hæmatoxylin solution as above for ten minutes; then remove the excess of hæmatoxylin fluid from the sections by means of blotting-paper, and bring the series for five to ten minutes into 70 per cent. alcohol containing only a few drops of a strong (5 per cent.) solution of bichromate. This must be done in the dark. If the hæmatoxylin be not removed with blotting-paper as described, the celloidin will take the stain. The sections should appear steel-blue to steel-grey.

195. Mallory's **Phospho-molybdic Acid Hæmatoxylin** (*Anat. Anzeig.*, 1891, p. 375 ; see also *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 341).—One part of 10 per cent. phospho-molybdic acid solution, 1 part hæmatoxylin, 100 parts water, and 6 to 10 parts chloral hydrate. Let the solution ripen for a week in sunlight, and filter. This stain is recommended for preparations of central nervous system, but may perhaps be useful in other cases. Sections should be stained for from 10 minutes to 1 hour, and washed out in two or three changes of 40 to 50 per cent. alcohol. Dehydrate and mount as usual. The celloidin remains colourless. The stain is blue, and in its general effect something like a nigrosin stain. Besides ganglion-cells and glia-cells, axis-cylinders are stained. It is necessary that the solution be *saturated* with hæmatoxylin in order to obtain the best results ; if a good stain be not obtained at once, more hæmatoxylin must be added.

196. Iron Hæmatoxylin (Bütschli, *Unters. über mikrosko-*

pische Schäume u. das Protoplasma, &c., 1892; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 197).—Sections treated with a weak brown aqueous solution of ferric acetate, washed with water, and stained in 0·5 per cent. aqueous solution of hæmatoxylin. This treatment gives a blue-black or brown-black stain of extraordinary intensity. The process was used by Bütschli for staining sections, 1 μ in thickness, of Protozoa.

197. Iron Hæmatoxylin (M. Heidenhain, "Über Kern und Protoplasma," in *Festschr. Herrn Geheimr. A. v. Kölliker, &c., gewidm.*, 1892, p. 118; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 204). —Sections are treated from half an hour to at most two or three hours with a 1·5 to 4 per cent. solution of ammonio-sulphate of iron: $(NH_4)_2Fe_2(SO_4)_4$. They are then washed with water, and stained for half an hour to twelve hours in an aqueous solution (of about 0·5 per cent.) of hæmatoxylin. They are then rinsed with water, and again treated with the iron solution, which slowly washes out the stain. As soon as a satisfactory differentiation has been obtained, the preparations are washed for a quarter of an hour in running water, and mounted. The results differ according to the duration of the treatment with the iron and the stain. If the baths have been of short duration, viz. not more than half an hour in the iron and as much in the stain, *blue* preparations will be obtained. These show a very intense and highly differentiated stain of all nuclear structures, achromatic as well as chromatic, cytoplasmic structures being pale. If the baths in the iron and in the stain have been prolonged, and the subsequent differentiation in the second iron bath also duly prolonged, *black* preparations will result. These show chromosomes stained, central and polar bodies stained intensely black, cytoplasm sometimes colourless, sometimes grey, in which case achromatic spindle-fibres and cell-plates are stained, connective-tissue fibres black, red blood-corpuscles black, micro-organisms sharply stained.

198. Weigert's Hæmatoxylin.—This method, which is the inverse of Heidenhain's (§ 193), is only applicable to nerve-tissues, and therefore will be described in the chapter on "Nerve Methods" in Part II.

199. Minot's Hæmatoxylin Methods (*Zeit. f. wiss. Mik.*, iii, 2, 1886,

p. 177).—Minot's account of these is as follows :—" They may be employed with sections of tissues hardened in various ways, and need not be confined to Müller's fluid or chromic acid specimens (as in Weigert's process). The sections are soaked first in a salt solution for ten to fifteen minutes. The following aqueous salt solutions seem to be the most valuable:—Alum, 2 per cent. ; chromic acid, 1 per cent. ; bichromate of potassium, 5 per cent. ; acetate of copper, nearly saturated. After soaking in one of these, the section is passed quickly through distilled water, and placed at once in Weigert's hæmatoxylin (1 part of the crystals in 10 parts alcohol *plus* 90 parts water), and may be left a short time for direct coloration, then washed and mounted, or a longer time until they become black, and are to be washed out by Weigert's iron solution (water 100, borax 2, ferricyanide of potassium $2\frac{1}{2}$). The sections ought to be moved about constantly in the iron solution, otherwise the colour will be extracted irregularly. The copper hæmatoxylin goes out very rapidly, so that with that stain it is better to dilute the iron solution with twice its bulk of water before placing the sections in it. After the iron solution the sections must be washed very thoroughly in water, to avoid further fading out, from which one is not entirely secure until the sections are actually mounted in balsam.

"These methods are all merely modifications of Weigert's, Heidenhain's, and Böhmer's methods."

D. *Other Organic Stains.*

200. Alizarin, so far as I am aware, is only used for Nerve-Centres. See Part II.

201. Purpurin is useful for cartilage and muscle. It is soluble in a boiling aqueous solution of alum, from which it normally precipitates on cooling, but may be prevented from doing so by the addition of a certain proportion of alcohol. The employment of an alum solution as a vehicle for the colouring matter has the advantage, at least so far as cartilage is concerned, of fixing the cellular elements at the same time that they are stained. (Ranvier found that alum in a solution of 5—1000 was the best of all fixing agents for cartilage-cells—*Traité*, p. 279.)

RANVIER'S *formula* (*Traité technique*, p. 280).—200 grms. of water and 1 of alum are boiled in a porcelain capsule ; purpurin rubbed up in water is added, and the boiling continued. The purpurin being dissolved to saturation (this is ensured by taking care to have an undissolved excess in the capsule), the solution is filtered hot into a flask containing 60 c.c. of alcohol (36° Cartier = 90 per cent.).

There is thus obtained a solution of an orange-rose colour, presenting a marked degree of fluorescence.

(As regards the quantity of alcohol to be taken, Duval writes that it should always be one fourth in volume of the total mixture—*Précis de Technique histologique*, p. 221.)

The solution does not keep well for more than a few weeks.

Sections of fresh cartilage are to be placed in a small quantity (only a few cubic centimetres) of the solution, and after remaining there twenty-four to forty-eight hours are washed in water and mounted in glycerin. The stain is nuclear, the matrix remaining almost colourless. Duval (loc. cit.) states that this stain has a special selective action on sections of central nervous system (especially spinal cord) obtained from tissues hardened in bichromate of ammonia (2—1000), and mounted, after staining for forty-eight hours, in Canada balsam. The nerve cells and processes, axis-cylinders, and fibres of connective tissue are unstained; but the nuclei of connective tissue and of the capillaries are stained red.

Grenacher's formula (*Arch. f. mik. Anat.*, xvi, 1879, p. 470). —In 50 cubic centimetres of glycerin (pure or diluted with very little water) dissolve from 1 to 3 per cent. powdered alum; add a knife-pointful of purpurin, and boil. (Alcohol must *not* be added.) Let the orange-coloured fluorescent solution stand for two or three days, and then filter.

A nuclear stain; ten to thirty minutes generally suffice to produce good staining. The solution is stable, which Grenacher finds that Ranvier's solution is not, the latter precipitating after a few days.

202. Indigo.—Indigo is employed in histology in the form of solutions of so-called indigo-carmine, or sulphindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, and is therefore not capable of being usefully employed *alone*. It is, however, of great use when employed to bring about a *double* stain in conjunction with carmine. Though it has no selective preference for nuclei or protoplasm, it possesses to a high degree the property of imparting different hues and intensity of stain to different tissues; and the nuclei being brought out by carmine, preparations are obtained of a diagrammatic clearness that is not afforded by carmine alone.

Indigo-carmine is found in commerce. The reader who may desire to prepare it himself will find the necessary directions in *Arch. f. mik. Anat.*, x, 1874, p. 32, and in *Journ. Roy. Micr. Soc.*, ii, 1879, p. 614.

203. Thiersch's Oxalic Acid Indigo-carmine (see *Arch. f. mik. Anat.*, i, 1865, p. 150).

204. Tincture of Saffron (H. Blanc, *Zool. Anzeig.*, 129, 1883, p. 23).

—Dissolve 5 grms. of saffron in 15 c.c. of absolute alcohol; allow the solution to settle for a few days, filter, and dilute with one half of water. After staining wash out to the desired degree with 80 per cent. alcohol, then dehydrate with absolute alcohol, and mount in balsam.

205. Leven (*The Microscope*, ix, 1889, p. 88; *Journ. Roy. Mic. Soc.*, 1889, p. 467) has been using saffron for the study of the regeneration of muscle. He stains in a solution containing saffron 1 part, absolute alcohol 100 parts, water 200, and washes out in acidulated alcohol (0·5 per cent. HCl). Karyokinetic figures dark red, muscle nuclei pale with dark red nucleoli. Leucocytes stain strongly.

206. Orchella (Orseille) (Wedl, *Arch. f. path. Anat.*, lxxiv, p. 143; *Journ. Roy. Mic. Soc.*, ii, 1879: for an account of this substance *vide* Cooley's *Cyclopædia, sub voce* "Archil"). —French orchella extract, from which the excess of ammonia has been removed by gentle warming in a sand-bath, is poured into a mixture of absolute alcohol 20 c.c., acetic acid (concentrated, of 1·070 sp. gr.) 5 c.c., and water 40 c.c., until a saturated dark red stain is obtained, which must then be filtered once or twice. Sections are washed with water, drained, and treated with the stain. Mount in levulose. A protoplasmic stain, nuclei remaining colourless. Connective-tissue cells stain deeply, the intercellular substance less deeply. Epithelia, if horny or calcareous, are not stained. The basic substance of bone and teeth take the stain, and so do ganglion-cells and their processes.

This colour ought to be useful for double-staining. Fol (*Lehrb.*, p. 192) advises staining for an hour in Wedl's solution, then rinsing with alcohol, and staining in a complementary stain.

207. Orceïn (Israël, *Virchow's Archiv*, cv, 1886, p. 169; *Journ. Roy. Mic. Soc.*, 1887, p. 514).—Orceïn (not "orcin," as erroneously printed in the last edition of this work) is a vegetable dye obtained from tinctorial lichens, which unites in itself the staining properties of the basic and acid stains, and also the combination of two contrast colours. Israël stains sections in a saturated acetic acid solution, washes in distilled water, and passes rapidly through absolute alcohol to thick cedar oil, in which the preparations remain definitively mounted. Nuclei blue, protoplasm red.

For the specific staining of elastic tissue by means of this reagent, a subject which has been carefully worked out by Tänzer and Unna, see the paragraphs on the Connective Tissues in Part II.

208. Kernschwarz (Platner, *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 350; *Journ. Roy. Mic. Soc.*, 1888, p. 675).—Kernschwarz

is a black liquid of unknown composition, prepared in Russia. It may be obtained from Grübler (address § 94). Sections (sections only, this colour behaving like safranin, for instance) may be stained in a tolerably strong dilution of the concentrated liquid, and washed out (it may be for some hours) in an alkaline aqueous liquid. Dilute ammonia will do, but it is better to take a not quite saturated solution of carbonate of lithium (you may take a saturated solution, and dilute it with three, four, or more volumes of water). The result is a nuclear stain in the cytological sense; nuclear figures of division are stained deeply, resting chromatin less deeply or not at all, cytoplasm unstained or faintly grey. A peculiarity of this stain, on which much stress was laid by Platner in his first announcement of the colour, is that it stains also the *Nebenkern* (which safranin and the other anilins do not do when employed in the usual way).

After some experimentation I feel bound to say that I do not think Kernschwarz has the importance seemingly claimed for it by Platner. It certainly stains the Nebenkern, but it does so less rather than more effectively than hæmatoxylin, not to mention Henneguy's permanganate-safranin method (§ 106), or Flemming's orange method, § 258. Platner seems to have come to this conclusion himself, his latest work on the Nebenkern making no further mention of Kernschwarz, and having been done apparently entirely with hæmatoxylin. And, as a nuclear stain, Kernschwarz seems to me inferior to hæmatoxylin, to say nothing of the chromatin-staining anilins. Only in the event of its being found available for staining in the mass (which may be possible, though I have not succeeded in it) will Kernschwarz, as it seems to me, be found to be an important reagent.

209. Other Stains.—Litmus, red cabbage, bilberry juice ("myrtillus"), black currant juice ("ribésine"), and walnut juice ("nucina") have been recommended by Lawson Tait, Lavdowsky, Fol, and Léon respectively. They do not appear to call for further notice here.

CHAPTER XII.

METALLIC STAINS (IMPREGNATION METHODS).

210. The Characters of Impregnation-Stains.—By impregnation is understood a mode of coloration in which a colouring matter is deposited in certain elements of tissues in the form of a more or less finely granular or sometimes even flocculent *precipitate*—the impregnated elements becoming in consequence opaque. By staining, on the other hand, is understood a mode of coloration in which the colouring matter is retained by the tissues in a state of *solution*, showing no visible solid particles under the microscope, the stained elements remaining in consequence transparent. But it should be understood that it is not correct to draw a hard and fast line between the two kinds of coloration. Some of the metallic salts treated of in this chapter give, besides an impregnation, in some cases a true stain. And some of the dyes that have been treated of in preceding chapters give, besides a stain, a true impregnation. Methylen blue, for instance, will give in one and the same preparation an impregation and a stain; and critical examination of most fairly successful gold-chloride preparations will show that the coloration is in places of the nature of a finely divided solid deposit, in others a perfectly transparent stain.

Thus is justified the alternative title that this chapter has always borne—Metallic Stains, *or* Impregnation Methods. Impregnations are distinguished as *negative* and *positive*. In a negative impregnation, intercellular substances alone are coloured of a deep black or brown or violet, according to the method employed; the cells themselves remaining colourless or very lightly tinted. In a positive impregnation the cells are stained and the intercellular spaces are unstained. (This explanation is the more needful as a directly contrary statement is made in a recent *Lehrbuch*.)

Negative impregnation is *primary* because it is brought about by the direct reduction of a metal in the intercellular spaces. Positive impregnation is *secondary* (in the case of silver nitrate) because it is brought about by the solution in the liquids of the tissues of the metallic deposit formed by a primary or negative impregnation, and the consequent staining of the cells by the new solution of metallic salt thus formed. These secondary impregnations take place when the reduction of the metal in the primary impregnation is not sufficiently energetic (see on these points His, *Schweizer Zeit. f. Heilk.*, ii, Heft 1, p. 1; Gierke, *Zeit. f. wiss. Mik.*, i, p. 393; Ranvier, *Traité*, p. 107).

There still exists considerable obscurity as to the nature of the black or brown deposit formed in the intercellular spaces in cases of primary impregnation with a silver salt; v. Recklinghausen held that the silver salt combined with a hypothetical intercellular cement-substance (*Kittsubstanz*), forming a compound that blackens under the influence of light. Other authors refuse to believe in the intercellular cement, and hold either that the coloured lines represent stained cell-membranes, or that the metallic salt combines with the albuminous and saline liquids that surround the cells, and is precipitated in simple intercellular *spaces*. Schwalbe (*Arch. f. mik. Anat.*, vi, 1870, p. 5) thinks that two cases should be distinguished; the *black* lines that are obtained by the action of very weak solutions for a very short time being due to a true precipitate formed by reduction of metal in the intercellular liquids, the *brown* lines that are obtained by exposing tissues for a longer period to the action of more concentrated solutions being due to the formation of a compound of metal and cement-substance that becomes brown on exposure to light. (For the history of these questions see Gierke's *Färberei zu mikroskopischen Zwecken*.)

211. Action of Light on Solutions of Metallic Salts.—Stock solutions of metallic salts are generally kept in the dark, or at least in coloured bottles, under the belief that exposure to light spoils them by precipitating the metal in a state of reduction. It has been pointed out above (§ 25) that in the case of osmic acid not light, but dust is the reducing agent; and that solutions may be exposed to light with impunity if

dust be absolutely denied access to them. I have now good evidence to the effect that the same is the case with other metallic solutions; and the interesting point is raised, whether such solutions are not positively improved for impregnation purposes by exposure to light! My able friend Dr. LINDSAY JOHNSON has been examining this question both from a histological and from a photographic point of view, and writes me as follows:

"One may (I find by experiment) state as a rule without exception that all the solutions of the chlorides and nitrates of the metals will keep indefinitely in clean white stoppered bottles in the sunlight; and as far as osmium, uranium, gold and silver, and platinum are concerned, actually improve or ripen by a good sunning. All photographers tell me their papers salt more evenly by old well-sunned silver nitrate than by a fresh solution kept in the dark; and I go so far as to say that this is one of the reasons why gold stains are so unsatisfactory." I have not been able myself to perform any experiments for the purpose of confirming the hypothesis of ripening by sunning, but I must say that it appears to me very plausible and deserving of further inquiry.

Silver.

212. Silver Nitrate: Generalities.—This is the most commonly used salt of silver. The general principles of its employment are so well stated by RANVIER (*Traité*, p. 105) that I cannot do better than abstract his account.

Silver nitrate may be employed either in solution or in the solid state. The latter method is the less frequently employed, but is easy and gives good results. It is useful for the study of the cornea and of fibrous tissue, but is not suitable for epithelia. For the cornea, for instance, proceed as follows:—The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water; it is then brushed with a camel's-hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. On subsequent examination it will be found that the silver nitrate, which was dissolved by the liquid that bathes the surface of the cornea, has traversed the epithelium and soaked

into the fibrous tissue, on the surface of which it is reduced by the action of light. The cells of the tissues will be found unstained.

Silver nitrate is generally employed in solution in the following manner:—A 1 per cent. solution is taken, to which two, three, or four volumes of water are added according to circumstances. The mode of employment varies in its details according to circumstances, a point which is very important to observe. In the case of a membrane such as the epiploön, the membrane must be stretched like a drum-head over a porcelain dish,* and washed with distilled water, in order to remove the albuminates and white blood-corpuscles that are found on its surface; it is then washed with the solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has become white, and has begun to turn of a blackish grey, the membrane is removed, washed in distilled water, and mounted on a slide in some suitable examination medium.

If the membrane were left in the water the cells would become detached, and would not be found in the finished preparation.

If the membrane had not been stretched as directed the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface, and the forms of the cells would be disguised.

If the membrane had not been washed with distilled water before impregnation there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that very often impurities in the specimen have been described as stomata of the tissue.

If the solution be taken too weak—for instance, 1·500 or 1·1000, or if the light be not brilliant—a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver.

* The Hoggans' histological rings, for which see below, § 216, will be found much more convenient.

In general, in a good "impregnation," the contents of cells, and especially nuclei, are quite invisible.

Ranvier notes that when tissues are to be impregnated by immersion they should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver, which would give rise to deceptive appearances.

Impregnation with silver may be followed by treatment with picro-carmine (or other carmine stain), which will bring out the nuclei, provided the impregnation has not been overdone.

It should be noticed that impregnations only succeed with *fresh* tissues, and cannot be made to succeed with tissues preserved in any way.

213. Silver Nitrate: the Solutions to be employed (RANVIER). —The solutions generally employed by Ranvier vary in strength from 1·300 to 1·500. Thus 1·300 is used for the epiploön, pulmonary endothelium, cartilage, tendon; whilst a strength of 1·500 is employed for the study of the phrenic centre, and for that of the epithelium of the intestine. For the impregnation of the endothelium of blood-vessels (by injection) solutions of 1·500 to 1·800 are taken.

M. DUVAL (*Précis*, p. 229) recommends solutions of 1, 2, or at most 3 per cent.

V. RECKLINGHAUSEN used, for the cornea, a strength of from 1—400 to 1—500 (*Die Lymphgefässe*, &c., Berlin, 1862, p. 5).

ROBINSKI (*Arch. de Physiol.*, 1869, p. 451) used solutions varying between 0·1 and 0·2 per cent., which he allowed to act for thirty seconds.

REICH (*Sitzb. d. wien. Akad.*, 1873, Abth. iii, April; *Zeit. f. wiss. Mik.*, i, p. 397) takes solutions of from 1—600 to 1—400 for the study of the endothelium of vessels by injection.

ROUGET (*Arch. de Physiol.*, 1873, p. 603) employed solutions as weak as 1—750, or even 1—1000, exposing the tissues to their action several times over, and washing them with water after each bath.

The HERTWIGS take, for marine animals, a 1 per cent. solution (*Jen. Zeit. f. Naturk.*, xvi, pp. 313 and 324).

The Hoggans (*Journ. of Anat. and Physiol.*, xv, 1881, p. 477) take, for lymphatics, a 1 per cent. solution.

Tourneux and Hermann (Robin's *Journal de l'Anat.*, 1876, p. 200), in their fine studies on the epithelia of Invertebrates, employed a solution of 3·1000 strength, and in some cases weaker solutions. The tissues were allowed to remain in the silver-bath for one hour, and were washed out with alcohol of 36° strength.

Hoyer (*Arch. f. mik. Anat.*, 1876, p. 649) takes a solution of nitrate of silver of known strength, and adds ammonia to it until the precipitate that is formed just redissolves, then dilutes the solution until it contains from 0·75 to 0·50 per cent. of the salt.

This *ammonio-nitrate* solution is intended principally for the impregnation of the endothelium of vessels by injection, but can also be used for the impregnation of membranes by pouring on. It has the advantage of impregnating absolutely nothing but endothelium or epithelium; connective tissue is not affected by it. It is also said to give a sharper localisation of the stain than the ordinary solutions.

Dekhuysen (*Anat. Anz.*, iv, 1889, No. 25, p. 789; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 351) has applied to tissues of terrestrial animals the method of Harmer for marine animals (see below, § 217). He washes a portion of mesentery of a frog in a 1·3 per cent. solution of nitrate of potash, and brings it for from three to six minutes into a 0·25 per cent. solution of silver nitrate containing 3 per cent. of nitric acid. After that time it is brought into pure 3 per cent. nitric acid, thence after a few minutes into 96 per cent. alcohol, and then into clove oil, in which it is reduced in diffused light in a few minutes. The method is stated to have the advantage of giving an excellent fixation of tissues, and of allowing a good nuclear after-stain with hæmatoxylin, safranin, or methyl green.

Alferow (*Arch. de Physiol.*, 1874; *Laboratoire d'histologie du Collège de France*, 1874, p. 258; Duval, *Précis*, p. 230) recommends the soluble silver salts of organic acids, viz. the picrate, lactate, acetate, and citrate, as giving better results than the nitrate. He employs them in solutions of 1·800, and adds to the solution employed for staining a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated

solution of the acid to 800 c.c. of the solution of the salt). The object of the free acid is to decompose the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues, leaving only the albuminate, which is a more resistent compound.

214. Silver Nitrate: Reduction.—Reduction may be effected in other media than distilled water.

V. Recklinghausen washed his preparations in salt solution before exposing them to the light in distilled water (*Arch. f. path. Anat.*, xix, p. 451). Physiological salt solution (0·75 per cent.) is commonly used for these washings.

Müller (*Arch. f. path. Anat.*, xxxi, p. 110), after impregnation by immersion for two or three minutes in a 1 per cent. solution of nitrate of silver in the dark, adds to the solution a small quantity of 1 per cent. solution of iodide of silver (dissolved by the aid of a little iodide of potassium). After being agitated in this mixture the preparations are washed with distilled water, and exposed to the light for two days in a 1 per cent. solution of nitrate of silver (see also Gierke, in *Zeit. f. wiss. Mik.*, i, 1884, p. 396).

Rouget (*Arch. de Physiol.*, 1873, p. 603) reduces in glycerin.

Sattler (*Arch. f. Mik. Anat.*, xxi, p. 672) exposes to the light for a few minutes in water acidulated with acetic or formic acid. Thanhoffer (*Das Mikroskop.*, 1880) recommends this method. He employs a 2 per cent. solution of acetic acid.

Krause brings his preparations, after washing, into a light red solution of permanganate of potash. Reduction takes place very quickly, even in the dark. The method does not always succeed (see Gierke, in *Zeit. f. wiss. Mik.*, i, 1884, p. 400).

Oppitz brings his preparations for two or three minutes into a 0·25 or 0·50 per cent. solution of chloride of tin. Reduction takes place very rapidly (Gierke, l. c.).

Jakimovitch (*Journ. de l'Anat.*, xxiii, 1888, p. 142; *Journ. Roy. Mic. Soc.*, 1889, p. 297) brings nerve preparations, as soon as they have become of a dark brown colour, into a mixture of formic acid 1 part, amyl alcohol 1 part, and water 100 parts. The objects exposed to the light in this mixture for two or three days at first become brighter, a part

of the reduced silver being dissolved; hence the mixture must be renewed from time to time. When all the silver has dissolved, a darker colour is permanently assumed. The nerve-cells are left in this mixture for five to seven days.

215. After-Blackening.—Legros (*Journ. de l'Anat.*, 1868, p. 275) washes his preparations after reduction in hyposulphite of soda, which prevents after-blackening. According to Duval (*Précis*, p. 230) they should be washed for a few seconds only in 2 per cent. solution, and then in distilled water.

216. The Hoggans' Histological Rings are vulcanite rings made in pairs, in which one ring just fits into the other, so as to clip and stretch pieces of membrane between them. They will be found described and figured in *Journ. Roy. Mic. Soc.*, ii, 1879, p. 357, and in Robin's *Journ. de l'Anat.*, 1879, p. 54. They may be obtained, in sets of various sizes (that of seven eighths of an inch being the most convenient for 3 × 1 slides), of Burge and Warren, 42, Kirby Street, Hatton Garden, London, E.C., price ten shillings the dozen pairs.

This useful little apparatus has lately been reinvented by Eternod (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 39), and is made according to his designs by Demaurex, Bandagiste, Fusterie, Geneva (Switzerland).

The Hoggans' histological rings were described by me in the first edition of this work, p. 375, and in the *Traité d. Méth. techn.*, Lee et Henneguy, p. 138; so that I am in no way responsible for the waste of time involved in this reinvention on the part of the inventor, and the editors of the *Zeit. f. wiss. Mik.* and other journals.

217. Silver Impregnation of Marine Animals.—On account of the considerable quantity of chlorides that bathe the tissues of marine animals, these cannot be treated *directly* with nitrate of silver.

Hertwig (*Jen. Zeit.*, xiv, 1880, p. 324) recommends fixing them with a weak solution of osmic acid, then washing with distilled water until the wash-water gives no more than an insignificant precipitate with silver nitrate, and then treating for six minutes with 1 per cent. solution of silver nitrate.

Harmer (*Mitth. Zool. Stat. Neapel*, v, 1884, pp. 44 to 56)

has discovered that many marine animals will live for some time (half an hour) in a 5 per cent. solution of nitrate of potash in distilled water. By washing them in this way they may be freed from the greater part of their chlorides, and may then be treated with silver nitrate in the usual way. This method gave good results with *Loxosoma* and *Pedicellina*, with Medusæ, Hydroids, *Sagitta* and *Appendicularia*.

Vosmaer has been able by this means to demonstrate the epithelium of *Chondrosia* and *Thenea*, which Sollas was unable to see; and Meyer has obtained good results with Annelids and ova of Teleostea. Few animals resist the action of nitrate of potash so well as *Loxosoma* and *Pedicellina*, but die in the solution in a few minutes. Their tissues, however, suffer but little change, and give good impregnations. Harmer thinks that for these animals other solutions having the same density as sea-water might be substituted for the nitrate of potash, and recommends a 4·5 per cent. solution of sulphate of soda.

See also Dekhuysen's method, *ante*, § 213.

218. Impregnation of Nerve Tissue.—For this subject, which includes the important bichromate-and-silver method of Golgi, see Part II.

219. Double-staining Silver-stained Tissues.—The nuclei of tissues impregnated with silver may be stained with the usual reagents, provided that solutions containing free ammonia be avoided, as this would dissolve out the silver. These stains will only succeed, however, with successful negative impregnations, as nuclei that have been impregnated will not take the second stain.

Impregnation with silver may be followed by impregnation with gold. In this case the gold generally substitutes itself for the silver in the tissues, and though the results are sharp and precise, the effect of a double stain is not produced.

Gold.

220. The Characters of Gold Impregnations.—Gold chloride differs from nitrate of silver in that it generally gives *positive* (§ 210) impregnations only. It only gives negative images, so far as I know, when caused to act on tissues that have first received a negative impregnation with silver, the gold substituting itself for the silver. In order to obtain these images

you first impregnate very lightly with silver; reduce; treat for a few minutes with a 0·5 per cent. solution of gold chloride, and reduce in acidulated distilled water.

This process, however, is in but little use, and except for the staining of cytoplasm for cytological researches and for certain special studies on the cornea, and on connective tissue, the almost exclusive function of gold chloride is the impregnation of nervous tissue. For this tissue, gold chloride exhibits a remarkable selectivity, in virtue of which it justly ranks as a most valuable reagent for the study of nerve end-organs and the distribution of nerves.

For all the objects above named gold chloride is capable of furnishing preparations that for beauty and clearness cannot be surpassed, if even they can be equalled by any other means. A successful gold preparation shows at a glance, with diagrammatic clearness, a wealth of minute detail which perhaps can only be painfully glimpsed by other means. But not every gold preparation *is* successful. I think there is no use in blinking the fact that very few are successful (one of the most experienced authorities in the matter told me lately that, as to nerve end-organs at all events, one preparation in ten thousand is successful). I took up in the first edition of this work the doubtless unpopular position that "with all possible precautions gold chloride is uncertain in its action, and that the results obtained by means of it need to be controlled by the employment of other methods," and illustrated that position at considerable length.

That this position was the correct one is now generally admitted. It is acknowledged to be abundantly evident that the very best gold preparations give images that are only worthy of credence as to what they show, and furnish absolutely no evidence whatever as to the non-existence of anything that they do not show; for you can never be sure that the imbibition of the salt has not capriciously failed, or its reduction capriciously stopped at any point. That the images frequently *do* stop capriciously short in the representation of reality there is abundant evidence. One such case has been treated by me *ex professo* in *Recueil Zool. Suisse*, i, 1884, p. 685 (*Les organes chordotonaux des Diptères, et la méthode du chlorure d'or*).

The authors of some of the methods about to be described

claim for them that they give permanent preparations. I warn the reader against indulging in the hope that, with all possible precautions, his preparations will retain all their beauty for more than a few weeks. A successful gold preparation is certainly a thing of beauty, but it is exactly the opposite of a joy for ever. The able histologist whose experience I have taken the liberty of quoting above tells me that "as to permanence, they are—

"'Like the snowfall on the river.'"

Still, the greater the care taken in preparation, and particularly the greater the care taken to ensure thorough reduction of the gold, the longer will be the life of the preparations.

Careful attention to the devices to this end detailed in the following paragraphs will do much; and possibly LINDSAY JOHNSON'S suggestion (*supra*, § 211) of the utility of "sunning" the solutions before use may prove an unexpected help.

221. As to the Commercial Salts of Gold.—It is necessary to remind the histologist that "all is not gold that glitters." Many things are not what they seem, and gold chloride is one of them, as will appear from the following quotation from SQUIRE'S *Methods and Formulæ*, &c. (p. 43), an excellent authority on the chemistry of histological reagents:

"Commercial chloride of gold is not the pure chloride, $AuCl_3$, but the crystallised double chloride of gold and sodium, containing 50 per cent. of metallic gold.

"Commercial chloride of gold and sodium is the above crystallised double chloride mixed with an equal weight of chloride of sodium, and contains 25 per cent. of metallic gold."

222. The Two Types of Method.—Gold methods may be divided into two groups. The one, chiefly concerned with the study of peripheral nerves or nerve end-organs, is characterised by employing either perfectly fresh tissues or tissues that have been subjected to a special treatment by organic acids; the other, concerned with the study of nerve-centres, is characterised by the employment of tissues hardened in the usual way.

The hitherto classical rule, that for researches on nerve-

endings the tissues should be taken perfectly fresh, seems not to be valid for all cases. For DRASCH (*Sitzb. k. k. Acad. Wiss. Wien*, 1881, p. 171, and 1884, p. 516; and *Abhand. math.-phys. Cl. d. K. Sach. Ges. d. Wiss.*, xiv, No. 5, 1887; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492) finds that better results are obtained with tissue that have been allowed to lie after death for twelve, twenty-four, or even forty-eight hours in a cool place. He even suspects that the function of the organic acids in the methods inspired by Löwit's method, is to bring the tissues into somewhat the state in which they are naturally found at a certain moment of post-mortem process—a state, namely, in which the nerves have a special susceptibility for impregnation with gold.

223. Cohnheim's Method (*Virchow's Arch.*, Bd. xxxviii, pp. 346—349; *Stricker's Handb.*, p. 1100).—This, the archetype of the gold methods, was as follows:—Fresh pieces of cornea (or other tissue to be operated on) are put into solution of chloride of gold of 0·5 per cent. strength until they are thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin.

The method in this, its primitive form, often gave splendid results, but was very uncertain, giving sometimes a nuclear or protoplasmic stain, sometimes an extra-cellular impregnation similar to that of nitrate of silver. And the preparations thus obtained are anything but permanent.

224. Löwit's Method.—The principle of this process is that in order to facilitate the penetration of the gold and its subsequent reduction in the tissues, the tissues are made to swell up by treatment with formic acid before being brought into the gold-bath, and formic acid is employed to assist the reduction after impregnation.

The following directions as to this method, which may serve as a type of the modern methods of research on nerve-endings, are taken from FISCHER'S paper on the corpuscles of Meissner (*Arch. f. mik. Anat.*, xii, 1875, p. 366).

Löwit's method was first published by him in the *Wien. Sitzgsber.*, Bd. lxxi, Abth. iii, 1875, p. 1.

Small pieces of *fresh* skin are put into dilute formic acid

(one volume of water to one of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold-chloride solution (1½ per cent. to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1—3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark.) Thin sections are then made and mounted in dammar or glycerin. Successful preparations show the nerves alone stained, but it is not possible always to control the results.

225. Ranvier's Formic Acid Method (*Quart. Journ. Mic. Sci.* [N.S.], lxxx, 1880, p. 456).—The method of Löwit has been modified by many workers by omitting the final treatment with undiluted formic acid, and also in some other details. Ranvier proceeds as follows:—Reflecting that the action of the one third formic acid in which Löwit placed his tissues must be hurtful to the final ramifications of the nerves, he combines the formic acid with a fixing agent designed to antagonise its altering action, and takes for this purpose the chloride of gold itself. The tissues are placed in a mixture of chloride of gold and formic acid (4 parts of 1 per cent. gold chloride to 1 part of formic acid) which has been boiled and allowed to cool (Ranvier's *Traité*, p. 826). They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours); the reduction of the gold is effected either by the action of daylight in acidulated water, or in the dark in dilute formic acid (1 part of the acid to 4 parts of water).

The object of boiling the mixture of gold chloride and formic acid is this, that "by boiling in the presence of the acid the gold acquires a great tendency to reduction, and for this reason its selective action on nervous tissues is enhanced."

226. Ranvier's Lemon-juice Method (*Traité*, p. 813).—Instead of combining the formic acid with gold chloride in order to mitigate its action, recourse may be had to a less injurious acid than formic acid. Ranvier finds that of all acids lemon-juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon-juice, filtered through flannel, until they become transparent (five or ten minutes in the case

of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a bottle containing 50 c.c. of distilled water and two drops of acetic acid. They are exposed to the light, and the reduction is complete in twenty-four or forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent on account of their over-blackening with time, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water). The reduction is complete in twenty-four hours.

227. Viallanes' Osmic Acid Method (*Hist. et dév. des Insectes*, 1883, p. 42).—The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with one fourth formic acid for ten minutes; they are then put into solution of chloride of gold of 1·5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in one fourth formic acid. According to my experience this is a very excellent method, both the fixation by osmic acid and the great dilution of the gold solution being features likely to be of advantage in many cases.

228. Other Methods.—The numerous other methods that have been proposed differ from the foregoing partly in respect of the solutions used for impregnation, but chiefly in respect of details imagined for the purpose of facilitating the reduction of the gold, and rendering it as complete as possible.

Thus BASTIAN modified Cohnheim's original method by employing a solution of gold chloride of a strength of 1 to 2000, acidulated with HCl (1 drop to 75 c.c.), and performing the reduction in a mixture of equal parts of formic acid and water, *kept warm*, heat being an agent that furthers reduction.

HÉNOCQUE (*Arch. de l'Anat. et de la Physiol.*, 1870, p. 111) impregnates in a 0·5 per cent. solution of gold chloride, washes in water for twelve to twenty-four hours, and reduces, with the aid of heat, in a nearly saturated solution of tartaric acid. The tartaric acid solution must be contained in a well-stoppered bottle. The best temperature for reduction is 40°

to 50° C. Reduction is effected very rapidly, sometimes in a quarter of an hour.

This process has been described as the method of CHRSCHTSCHONOWIC (*Arch. f. mik. Anat.*, vii, 1872, p. 383).

HOYER (*Arch. f. mik. Anat.*, ix, 1873, p. 222) proceeds as follows :—(For corneal nerves.) The double chloride of gold and potassium has the following advantages over the simple gold chloride. It is more easy to be obtained of unvarying composition, it is more perfectly neutral, and its solutions are more perfectly stable. It is used in solutions of the same strength as chloride of gold, viz. 0·5 per cent. Corneæ must be very thoroughly imbibed with the solution. Small corneæ (rabbit, guinea-pig) require half to one hour, human corneæ two to five hours (in an acidulated solution). It is better to err on the side of too prolonged immersion rather than the contrary. In order to demonstrate the intra-epithelial ramifications of nerves, the gold is partially reduced by exposure for sixteen to twenty-four hours in (one or two ounces of) distilled water, and there is added to the water one or two drops of a pyrogallic acid developing solution, such as is used in photography (*vide* GERLACH, *Die Photographie als Hülfsmittel der mikroskopischen Forschung*, Leipzig, 1863). Or instead of treating them with the developing solution, the corneæ may be removed to a warm concentrated solution of tartaric acid, and remain there at the temperature of an incubating stove until the gold is fully reduced.

I have myself used the double chloride of gold and sodium with good results.

CIACCIO (*Journ. de Microgr.*, vii, 1883, p. 38 ; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 290) prefers the double chloride of gold and cadmium.

GERLACH, whose preparations of nerve-centres are said not to have been equalled since, proceeded as follows (Stricker's *Handb.*, 1872, p. 678) :—Spinal cord is hardened for fifteen to twenty days in a 1 to 2 per cent. solution of bichromate of ammonia. Thin sections are made and thrown into a solution of 1 part of double chloride of gold and potassium to 10,000 parts water, which is very slightly acidulated with HCl. They remain there from ten to twelve hours, and having become slightly violet, are washed in hydrochloric acid of 1 to 2 : 3000 strength, then brought for ten minutes into a mixture of 1

part HCl to 1000 parts of 60 per cent. alcohol, then for a few minutes into absolute alcohol, and thence into clove oil, for mounting in balsam.

Flechsig (*Die Leitungsbahnen im Gehirn*, 1876; *Arch. f. Anat. u. Phys.*, 1884, p. 453) reduces in a 10 per cent. solution of caustic soda.

Nesteroffsky treats impregated preparations with a drop of sulphydrate of ammonium, and finishes the reduction in glycerin (quoted from Gierke's *Färberei z. mik. Zwecken*).

Böhm reduces in Pritchard's solution.

Pritchard's solution consists of amyl alcohol, 1 per cent.; formic acid, 1 per cent.; and water, 98 per cent.

Manfredi treats fresh tissues as follows (*Arch. per le Sci. med.*, v, No. 15) :—Gold chloride, 1 per cent., half an hour; oxalic acid, 0·5 per cent.; they are then warmed in a water-bath to 36°, allowed to cool, and examined. Mount in glycerin. Sunny weather is necessary.

He treats tissues previously hardened in 2 per cent. solution of bichromate of potash, as follows (*ibid.*). They are put for half an hour into solution of arsenic acid, or into 1 per cent. acetic acid. They are then put into 1 per cent. gold chloride for half an hour, washed in water, and reduced in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

Boccardi (*Lavori Istit. Fisiol. Napoli*, 1886, i, p. 27; *Journ. Roy. Mic. Soc.*, 1888, p. 155) recommends oxalic acid of 0·1 per cent. or of 0·25 to 0·3 per cent., or a mixture of 5 c.c. pure formic acid, 1 c.c. of 1 per cent. oxalic acid, and 25 c.c. of water. Objects should remain in this fluid in the dark not longer than two to four hours.

Kolossow (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 52) impregnates for two or three hours in a 1 per cent. solution of gold chloride acidulated with 1 per cent. of HCl, and reduces for two or three days in the dark in a 0·01 per cent. to 0·02 per cent. solution of chromic acid.

Underwood (*Journ. Brit. Dental Ass.*, xi, 1890, p. 696; *Journ. Roy. Mic. Soc.*, 1890, p. 815) gives the following :—Wash sections in bicarbonate of soda (strength not given); treat them for half an hour to an hour with 1 per cent. solution of chloride of gold, neutralised if acid by bicarbonate of

soda; wash in water; reduce until the sections turn crimson (about an hour) in 1 per cent. formic acid kept "fairly hot" on a water-bath, in the dark; wash for half an hour in cold water; dry the sections (*sic*) and mount them in glycerin jelly ("specimens mounted in balsam always go wrong").

Dr. LINDSAY JOHNSON writes to me that besides the "sunning" of the impregnating solution recommended above (§ 211), the following precautions should be taken:—"The tissue must be well washed in distilled water, and the gold carefully acidulated with a neutral acetate or formiate, or acetic or formic acid, at least twenty-four hours before using; and then afterwards the tissue must be washed until no reaction occurs to test-paper."

For the details of the application of the methods of which the principles have been set forth above, and for those of the important processes of impregnation of central nerve organs, the reader is referred to those chapters of Part II. which treat of nerve-tissues and organs.

229. Ulterior Treatment of Impregnated Preparations.—Preparations may be mounted either in balsam or in acidulated glycerin (1 per cent. formic acid).

Theoretically they ought to be permanent if the reduction of the metal has been completed effected.

In practice, all are doomed to destruction in course of time by after-blackening, and few will be found to survive more than a few months. Ranvier states that this can be avoided by putting the preparations for a few days into alcohol, which possesses the property of stopping the reduction of the gold. But this must be taken to mean that by this means the period of usefulness of the preparations may be prolonged for some time, not indefinitely.

Blackened preparations may be *bleached* with cyanide or ferrocyanide of potassium. REDDING employs a weak solution of ferrocyanide; CYBULSKY a 0·5 per cent. solution of cyanide. But the results are far from being perfectly satisfactory.

Preparations may be double-stained with the usual stains (safranin and methyl green being very much to be recommended), but nuclei will only take the second stain in the case of negative impregnation.

230. Impregnation of Marine Animals.—For some reason that

I am unable to explain, the tissues of marine animals do not readily impregnate with gold in the fresh state. It is said by Fol that impregnation succeeds better with spirit specimens.

Other Metallic Stains.

231. Perchloride of Iron.—This reagent, introduced by Polaillon (*Journ. de l'Anat.*, iii, 1866, p. 43), sometimes gives most useful results, especially in the study of peripheral nerve-ganglia, in which it stains the nervous tissue alone, the connective tissue remaining colourless. The method consists in impregnating in perchloride of iron, and reducing in tannic, gallic, or pyrogallic acid.

The Hoggans, who have done very good work with this reagent, proceed as follows (*Journ. Quekett Club*, 1876; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 358):—The tissue (having been first fixed with silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit. It is then treated with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

Fol (see *ante*, § 52) fixes in perchloride solution, and treats the preparations for twenty-four hours with alcohol containing a trace of gallic acid.

Polaillon (l. c.) reduces in tannic acid.

This method is not applicable to chromic objects.

I should add that in my own experience I have found it very useful in certain special cases.

232. Pyrogallate of Iron (Roosevelt, *Med. Rec.*, ii, 1887, p. 84; *Journ. Roy. Mic. Soc.*, 1888, p. 157).—A stain composed of 20 drops of saturated solution of iron sulphate, 30 grms. water, and 15 to 20 drops pyrogallic acid.

233. Osmic Acid.—Everybody knows that osmic acid stains tissues. Most people, I should think, would be heartily glad if it did not. Meanwhile, to make the best of this willy-nilly stain, you may sometimes find it useful to treat the tissues with weak pyrogallic acid, which will very quickly turn them of a fine greenish black, sometimes giving useful differentiations.

This method was first published by me in 1887 (*La Cellule*, t. iv, fasc. 1, p. 110), and I have since republished it twice, with qualified recommendation. I have found it occasionally very useful, but have hesitated to recommend it for general work because I have usually found the stain too energetic and too little discriminating. It frequently happens that everything in a preparation comes out blacker than ink. The process has lately been re-invented by Kolossow (*Zeit. f. wiss. Mik.*, ix, 1892, p. 38) in a somewhat complicated form. He finds that the reaction obtained as I have described is not energetic enough, and recommends treating first with the osmium mixture given in § 28, and then with an elaborate "developing" mixture composed of water, alcohol, glycerin, tannin, and pyrogallic acid. For the reasons above stated, I fail to see the utility of this modification of the method (Kolossow himself

says that a simple 5 per cent. aqueous solution of tannin gives excellent results, sometimes superior to those obtained by the "developer"). See my note in *Zeit. f. wiss. Mik.*, ix, 1892, p. 185; also *op. cit.*, p. 316.

HERMANN (*Arch. f. Mik. Anat.*, xxxvii, 1891, p. 569; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 367) has been using with success crude pyroligneous acid for reducing the osmium in preparations fixed in his platino-aceto-osmic mixture (§ 36A). The acid should be allowed to act for twelve to eighteen hours.

ALLEN (*Quart. Journ. Mic. Sci.*, xxxiv, 1892, p. 75) has been treating in the same way objects fixed in FLEMMING'S solution, and attributes the method to VON MÄHRENTHAL.

The method of BRÖSICKE (*Centralb. f. d. med. Wiss.*, 1879, p. 873; *Zeit. f. wiss. Mik.*, i, 1884, p. 409) consists in treating osmium objects for twenty-four hours with a solution of 1 part of oxalic acid in 15 parts of water. This gives a Burgundy-red stain. It is necessary that the objects be washed and brought into the oxalic acid as soon as possible after the treatment with osmium, as if the osmium has once begun to blacken them, the oxalic acid is powerless afterwards to redden them.

234. Palladium Chloride (F. E. SCHULTZE, see *ante*, 52 and 84). **Prussian Blue** (see LEBER, *Arch. f. Ophthalm.*, xiv, p. 300; RANVIER, *Traité*, p. 108). **Cupric Sulphate** (see LEBER, *ibid.*). **Lead Chromate** (see LEBER, *ibid.*). **Sulphides** (see LANDOIS, *Centralb. f. d. med. Wiss.*, 1885, No. 55; and GIERKE, in *Zeit. f. wiss. Mik.*, i, 1884, p. 497). **Molybdate of Ammonium (Merkel; Krause)** (see GIERKE, *Zeit. f. wiss. Mik.*, i, 1884, p. 96). **Impregnation with Fats, Altmann's Method** (see *post*, "Corrosion").

CHAPTER XIII.

COMBINATION STAINS.

235. The Classes of Multiple Stains.—I distinguish two classes of multiple stains. In the one a pure nuclear stain, taking effect on *all* the nuclei of *all* the tissues of a preparation, is combined with a stain taking effect on *all the extra-nuclear parts of all the tissues.* Borax-carmine followed by indigo-carmine is a typical example of such a combination. In the second class, a stain taking effect on the totality of the elements of any one tissue exclusively is combined with a stain or stains of another colour taking effect on the totality of the elements of the other tissues.

The first class, aiming at enhancing the usefulness of a pure nuclear stain by improving the definition of extra-nuclear parts, has a legitimate scientific end in view, and is capable of rendering service in research. It will, therefore, here be treated much more fully than the second class, which is composed of much less generally useful, and too frequently merely ornamental stains.

236. Picric Acid Combinations.—I follow Flemming (*Zeit. f. wiss. Mik.*, i, 1884, p. 360) in pointing out that picric acid is perhaps the most generally useful of all secondary stains. It gives useful plasma stains with most of the nuclear stains, and particularly with carmine and hæmatoxylin. It may be used with the most delicate of these stains, even the delicate coloration of alum-carmine being in no wise injured by it. The *modus operandi* is as simple as possible; it consists merely in adding picric acid to the alcohols employed for dehydrating the objects after staining with a nuclear stain.

Care must be taken in adding picric acid to alcohol acidulated with HCl (see *ante*, § 169); in fact, this practice had better in general be avoided, and the picric acid only added to the pure alcohol used *after* washing out.

Combinations having Carmine for a Primary Stain.

237. Picro-carmine.—Picro-carmine is a double stain, if care be taken not to wash out the picrin beyond the point desired.

238. Borax-carmine and Picro-Carmine.—A very beautiful and precise double stain may be obtained by means of this combination. I add to a watch-glassful of Grenacher's alcoholic borax-carmine a few drops of picro-carmine.

The mixture will precipitate in the course of a few hours, but the stain will be obtained nevertheless.

Baumgarten's borax-picro-carmine (§ 171).

239. Carmine and Picric Acid.—This combination has been already sufficiently explained above (see § 236).

Legal's and Gilson's alum-carmine and picric acid mixture (§ 157).

Either of these combinations may be further combined with hæmatoxylin, giving very rich differentiations. The stain referred to by Bolsius in *La Cellule,* viii, 1, 1891, p. 6, should be understood to be obtained by means of a mixture of equal parts of hæmatoxylin solution and alum-carmine, followed by treatment with picric acid, and not by means of hæmatoxylin and picric acid alone, as reported in *Journ. Roy. Mic. Soc.,* 1891, p. 828, which would not give the colorations there described.

240. Orth's Picro-lithium Carmine.—See the place quoted, § 166. I consider the stain to be superfluous.

241. Seiler's Carmine followed by Indigo-Carmine (*Am. Quart. Mic. Journ.*, i, 1879, p. 220; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 613).—Stain in borax-carmine, wash out with HCl alcohol, wash out the acid, and after-stain in an *extremely dilute* alcoholic solution of indigo-carmine.

I find this method gives good results when applied to sections, but very bad results if it be attempted to stain in the mass with the indigo. The indigo over-stains the superficial layers before it has penetrated to the deeper layers. The method may be considered to be superseded by the carmine and anilin blue method, *infra,* § 243, which is more convenient.

242. Merkel's Carmine and Indigo-Carmine in One Stain (Merkel, *Unters. a. d. anat. Anst. Rostock,* 1874; *Month. Mic. Journ.*, 1877, pp. 242 and 317).

(A) Take 2 grms. of carmine, 8 grms. of borax, and 128 c.c. of water (or half a drachm of carmine, two drachms of borax, and four ounces of water). Rub

up in a mortar, allow the fluid to stand some time, decant, filter, and keep in a stoppered bottle.

(B) Take 8 grms. of indigo-carmine, 8 grms. of borax, and 128 c.c. of water (or two drachms indigo-carmine, two drachms borax, and four ounces water). Mix, decant, filter, and preserve as before.

Before using, mix A and B in equal proportions.

The objects to be stained must be thin; all traces of chromic acid or chromates must have been carefully washed out from them, and they must be soaked in alcohol before staining. Stain for fifteen or twenty minutes (MAX FLESCH finds it better to stain for several hours, see *Zeit. f. wiss. Mik.*, 1885, p. 350). Wash out with saturated aqueous solution of oxalic acid for a rather shorter time; wash the acid out with water, and mount as desired.

The oxalic acid is necessary for fixing the indigo-carmine, which, being very soluble in water, would otherwise be washed out. Unfortunately it precipitates carmine, so that successful preparations are not easily obtained, the carmine being generally either precipitated or turned into a straw-colour.

Authors (MERKEL, *loc. cit.*; NORRIS and SHAKESPEARE, *Amer. Journ. Med. Sc.*, January, 1877; MERKEL, *Mon. Mic. Journ.*, 1877, p. 242; MARSH, *Section Cutting*, p. 85; BAYERL, *Arch. f. mik. Anat.*, xxiii, 1885, pp. 36, 37; MACALLUM, *Trans. Canad. Instit.*, ii, 1892, p. 222; *Journ. Roy. Mic. Soc.*, 5, 1892, p. 698) are unanimous in stating that successful preparations show a most richly differentiated and yet very precise colouring. According to Bayerl, the stain is quite specifically elective for red blood-corpuscles, which are stained of an apple-green. The ground substance of cartilage and bone stains blue, their cells red.

The stain is not perfectly permanent. Bayerl recommends that benzin be used for clearing, in lieu of clove oil, which oxidises the stain and injures it.

This method has recently been recommended for nerve-centres. For Bayerl's application of it to ossifying cartilage see Part II.

I have put this method in small type because, though admirable for certain special purposes, it is not at all to be recommended for general work.

243. Carmine and Anilin Blue (or Bleu Lumière, or Bleu de Lyon).—DUVAL (*Précis de technique microscopique*, 1878, p. 225) proceeds as follows:—Stain with carmine "in the ordinary way;" dehydrate, and stain for a few minutes (ten minutes for a section of nerve-centres) in an alcoholic solution of anilin blue (ten drops of saturated solution of anilin blue soluble in alcohol to ten grammes of absolute alcohol, for sections of nerve-centres). Clear with turpentine, without further treatment with alcohol, and mount in balsam.

The sections should appear of a fine dark violet when taken from the anilin; they are extremely transparent under the microscope—nerve-cells and axis-cylinders reddish violet, blood-vessels bluish violet, and so sharply marked out that

the preparations have the aspect of injections. The connective elements are stained of a nearly pure blue, so that it is easy to distinguish them from the nervous elements.

Applicable to all kinds of tissues, but especially to sections of nerve-centres.

Recent authors recommend, instead of anilin blue, bleu de Lyon, dissolved in 70 per cent. alcohol acidulated with acetic acid (MAURICE and SCHULGIN), or bleu lumière, which has hardly any effect on nuclei.

The solutions of both these colours should be extremely dilute. They may be used for staining in the mass.

244. Carmine and Malachite Green.—MAAS (*Zeit. f. wiss. Zool.*, x, 4, 1890, p. 527 ; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 205) recommends borax-carmine followed by weak alcoholic solution of malachite green, with a final washing out with stronger alcohol.

245. Carmine and Methyl Green (MAX FLESCH, *Zool. Anz.*, 123, 1882, p. 554).—Sections of cartilage, skin, and glands, made from tissues hardened in Müller's solution and alcohol, were stained with picro-carmine, and subsequently (not "previously," as erroneously stated in *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 883) with an aqueous solution of commercial methyl green made of such a strength that the sections are just distinguishable in a watch-glassful of the solution when placed on a light ground.

The method is easy, gives good differentiations, but the stain does not appear likely to be permanent. Mount in balsam.

246. Picro-carmine and Iodine Green (STIRLING, *Journ. Anat. and Physiol.*, xv, 1881, p. 349, *et seq.*).

Iodine green has a specific action on adenoid tissue and mucous glands, which it stains of a bright green. But as methyl green has a similar action, the method appears to be in general superfluous. See "Mucus Glands," in Part II.

247. Picro-carmine, Rosein, and Anilin Blue; or Picro-carmine, Anilin Violet, and Anilin Blue; or Picro-carmine, Anilin Violet, and Iodine Green; or Picro-carmine, Rosein, and Iodine Green (HENEAGE GIBBES, *Journ. Roy. Mic. Soc.*, iii, 1880, p. 392).—H. Gibbes says of these methods that their great utility consists in their power of differentiating glandular structures according to their secretions. In a section of a dog's tongue "the ordinary mucous glands will be found to have taken on a purple colour, while the serous glands which supply the secretion to the taste-organs stain a totally different colour." (See also "Mucus Glands," in Part II.) Most of the differentiations aimed at in the last six sections will probably be better obtained by means of the EHRLICH-BIONDI stain given below, § 259.

248. Carmine and Picro-nigrosin (PIANESE), (see *Journ. Roy. Mic. Soc.*, 1892, p. 292).

249. Carmine and the Metallic Stains.—These combinations have been sufficiently spoken of in the passages devoted to gold and silver impregnation-methods. It will suffice here to call renewed attention to Zoltán v. Roboz, alum-carmine and osmic acid (§ 155).

Combinations having Hæmatoxylin for a Primary Stain.

250. Hæmatoxylin and Picric Acid.—This excellent combination has been treated of above (§ 236). See also § 239.

251. Hæmatoxylin and Eosin.—This is a well-known combination, and one of the most instructive that have yet been imagined, though many workers prefer the combination of hæmatoxylin with **Benzopurpurin** (§ 133). Objects may be stained with hæmatoxylin (either in the mass or as sections), and the sections stained for a few minutes in eosin. I think it is better to take the eosin weak, though it has been recommended (Stöhr, see *Zeit. f. wiss. Mik.*, i, 1884, p. 583) to take it saturated. Either aqueous or alcoholic solutions of eosin may be used.

This method is most particularly recommendable for embryological sections, as vitellus takes the eosin stain energetically, and so stands out boldly from the other germinal layers in which the blue of the hæmatoxylin dominates.

List (*Zeit. f. wiss. Mik.*, ii, 1885, p. 148) stains for twenty-four hours in a solution of three or four drops of Renaut's hæmatoxylic glycerin (§ 189) in 250 c.c. of water, and then for a few minutes in a mixture of one part of 0·5 per cent. aqueous solution of eosin with three parts of absolute alcohol.

Busch (*Verh. Berl. Phys. Ges.*, 1877; Gierke, *Zeit. f. wiss. Mik.*, i, 1884, p. 505) treats sections for some days with 0·5 per cent. solution of chromic acid, or 1 per cent. solution of bichromate of potash, washes, and stains first in an aqueous solution of eosin and then in hæmatoxylin. This process has been recommended for the study of the margin of ossification.

It should be noted that sections should be very well washed before being passed from eosin into hæmatoxylin or the reverse, as eosin very easily precipitates hæmatoxylin.

252. Renaut's Hæmatoxylic Eosin (Fol's *Lehrbuch*, p. 196).—Renaut has given from time to time several formulæ for this

stain. This one, communicated to Fol by Renaut, is the latest, and I suppress the others.

Take—

Concentrated aqueous solution of potassic eosin (*éosine à la potasse*) . . . . . 30 c.c.
Saturated solution of hæmatoxylin in alcohol (ought to have been kept some time and to have precipitated) . . . . . 40 c.c.
Saturated solution of potash alum in glycerin (of a density of about 1·26) . . . 130 c.c.

Mix, and let the mixture stand five or six weeks in a vessel covered with a sheet of paper pierced with holes until the alcohol is evaporated, then filter.

For staining, the solution may be used as it is or diluted. Staining goes on very slowly, and at first the colour is not held by the tissues, but disappears on washing. After some days or weeks, however, it becomes localised and fixed in the tissues. You may then mount in balsam, taking care to employ alcohol charged with a sufficient quantity of eosin. But it is frequently preferable to proceed by mounting the objects in the staining fluid diluted with one to two volumes of glycerin. After a few weeks this mounting medium will have become perfectly colourless through the absorption of the colour by the tissues.

The stain has a specific action on the cells of salivary and gastric glands. Mucus-cells become pale blue; salivary ferment-cells (crescent-cells of Gianuzzi) intense rose.

There is no doubt as to this being a very fine and useful stain. See also *Comptes Rendus*, 1879, p. 1039 (1re sér.), and *Arch. de Physiol.*, 1881, p. 640.

253. Hæmatoxylin and Benzopurpurin (Zschokke).—For this and the combination with **Delta Purpurin**, see *ante*, § 133.

254. Hæmatoxylin and Rubin and Orange.—Mr. A. Pringle informs me that this is an admirable combination. You should stain in Ehrlich's hæmatoxylin, wash in distilled or even acidified water, blue the stain with tap water, or water with a trace of alkali added to it, then stain in the rubin and orange.

255. Hæmatoxylin and Iodine Green (Stirling, *Journ. of Anat. and Physiol.*, xv, 1881, p. 353). **Hæmatoxylin and Nitrate of Rosa-**

nilin (List, *Zeit. f. wiss. Mik.*, ii, 1885, p. 149). See "Mucus-cells," in Part II.

256. Hæmatoxylin and Safranin.—This celebrated combination, which was used to such good effect by Rabl in his classical researches on nuclei (*Morph. Jahrb.*, x, 1884, p. 215), does not strictly belong to this subdivision, the safranin being the primary stain, though used after the hæmatoxylin. You stain *very lightly* with hæmatoxylin, so lightly that the stain would not be of any use by itself (Rabl uses very dilute Delafield's solution, for twenty-four hours); wash out first with water, and then with alcohol acidulated with HCl, then stain for some hours in (Pfitzner's) safranin, and wash out with pure alcohol. Rabl certainly was not far wrong when he wrote, "This method is unequalled by any other." For richness of detail in both nucleus and cytoplasm and tissue this method has indeed hardly been equalled.

Foà (*Festschr. R. Virchow gewidmet, &c.*, i, 1891, p. 481; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 227) prefers using the hæmatoxylin and safranin combined in one solution. You take—

Aq. dest.	ca. 100
Böhmer's hæmatoxylin	25
Safranin, usual 1 per cent. water and alcohol solution	20

Stain sections in a few drops of the mixture for from one to three minutes, wash in water, dehydrate, and mount; or before dehydrating treat with a weak alcoholic solution of picric acid, or, for some cases, of orange. This mixture was specially used for staining marrow in an investigation into the development of blood.

257. Hæmatoxylin and Metallic Stains. — The conditions under which hæmatoxylin can usefully be employed for staining impregnated tissues have been discussed under the heads of Gold and Silver. It only remains here to remind the reader that hæmatoxylin works very well after osmic acid.

Other Combinations.

258. The Anilin Double Stains.—These very important combinations are so numerous that only a small proportion of them can be mentioned here.

As regards *sections* stained by the indirect or Flemming's method, considerable latitude is allowable in the manipulations. Some persons stain first in the secondary stain (eosin, for instance), then wash and stain in the primary stain (by "primary" stain I constantly mean the nuclear stain), and wash out until the colour of the secondary stain reappears.

Another method, which will, I think, frequently be found preferable as allowing a stronger primary stain, is to stain first with the primary, say gentian violet, and then with the secondary, say eosin. Care must be taken not to wash out the first stain as completely as if you were going to mount it at once, else the operations required by the second stain may result in entirely removing the colour of the first.

Foremost amongst these processes must be mentioned Flemming's **Safranin, Gentian,** and **Orange Stain** (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 249; *ibid.*, p. 685; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 223, and viii, 3, p. 343).—This is a substitution method. You stain (for as much as two or three days if you want a very powerful stain) in strong alcoholic safranin solution diluted with anilin water (§ 101); rinse in distilled water; wash out in absolute alcohol, containing at most 0·1 per cent. of hydrochloric acid, until hardly any more colour comes away; stain for one to three hours in gentian (either a simple very strong aqueous solution, or, if you prefer, you may stain by the method of Gram, § 102); wash for a short time in distilled water; treat with concentrated, or at least fairly strong, aqueous solution of orange, which in virtue of its acid properties washes out most of the gentian. After at most a few minutes, whilst pale violet clouds are still being given off from the sections on agitation, bring them into absolute alcohol until hardly any more colour comes away, clear in clove or bergamot oil, and mount in damar or balsam before the last pale clouds of colour have ceased to come away (the orange should be the *orange G*, introduced into commerce by *Meister, Lucius,* and *Brüning*, of Höchst, and also prepared by the *Actiengesellschaft für Anilinfabrication*, Berlin; it may be obtained from Grübler, § 94).

This is not a triple stain in the sense of giving three different colours in the result; the orange does not act as a separate stain, but as an agent for the differentiation of the gentian stain. Chromatin and nucleoli are purple-red; achro-

matic spindle-fibrils grey to grey-brown, or, in very favorable cases, violet, and are very clearly brought out; attractive spheres, centrosomes, polar corpuscles, and "Zwischenkörper" reddish in light stains, brown-violet to black-brown in strong ones.

Lists of some colours that give good results as primary and as secondary stains are given in § 98. Besides the combinations there recommended I would particularly recommend that of **Gentian Violet** and **Eosin.** Stain and wash out (not too far) by Bizzozero's method (§ 102), and then stain for a few seconds, or as much as two to five minutes, in fairly strong aqueous solution of eosin, dehydrate rapidly with alcohol, clear, and mount; or **Benzopurpurin** may be taken instead of eosin (see § 133).

Attention should be paid to the decolourising action of the secondary stain on the primary (see § 98). This reaction may be utilised, as proposed by Baumgarten (*infra*, § 268). Resegotti (*Zeit. f. wiss. Mik.*, v, 3, 1888, p. 323) recommends staining for five minutes (this is for tissues fixed by alcohol) in strong solution of **Methyl Violet** or **Dahlia**, then washing out for one or two minutes in very weak solution of (alcoholic) **Eosin** or of **Säurefuchsin** in alcohol, then dehydrating and clearing.

In the following paragraphs are given some other methods of proved utility.

259. The Ehrlich-Biondi **Mixture** (or Ehrlich-Biondi-Heidenhain **Mixture**) (*Pflüger's Arch.*, xlii, 1888, p. 1; *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 520). This well-known stain is somewhat troublesome to prepare. It may be obtained ready made from Dr. Grübler (12, Baiersche Strasse, Leipzig).

The receipt is as follows:—To 100 c.c. saturated aqueous solution of Orange add with continual agitation 20 c.c. saturated aqueous solution of Säurefuchsin (Acid Fuchsin) and 50 c.c. of a like solution of Methyl Green.

Dilute the mixture with 60 to 100 volumes of water. The dilute solution ought to redden if acetic acid be added to it; and if a drop be placed on blotting-paper it should form a spot bluish green in the centre, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin.

Stain sections for six to twenty-four hours. Wash out with alcohol, clear with xylol, and mount in xylol balsam.

According to M. HEIDENHAIN's most recently published instructions ("Ueber Kern u. Protoplasma," in *Festschr. Herrn Geh. A. v. Kolliker gewidm.*, &c., 1892, p. 115; see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202) the Orange to be used should be "Orange G;" the Acid Fuchsin or Säurefuchsin should be "Rubin S" ("Rubin" is a synonym of Fuchsin); and the Methyl Green should be "Methylgrün OO." And it is *absolutely necessary* that these ingredients be those prepared under those names by the *Actienfabrik für Anilinfabrication in Berlin.*

The strong solutions directed to be taken readily precipitate on being mixed. To avoid this it is recommended by SQUIRE (*Methods and Formulæ*, &c., p. 37) to dilute them before mixing.

If (HEIDENHAIN, *Arch. f. mik. Anat.*, xxxv, 1890, p. 173; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 357) the alcohol used for washing out be slightly alkaline, the stain of the fuchsin will become relatively pale in the result, and the stain of the methyl green relatively strong; whilst a slightly acid reaction of the alcohol will produce the opposite effect. The energy of coloration of the fuchsin is often found to become weakened in kept solutions; it may be restored by adding very dilute acetic acid until a marked intensification of the red tint of the mixture is perceived. See § 639.

I have unfortunately not found time to make, as I should have wished, a thorough trial of this now favourite stain. But I have collected a good deal of trustworthy evidence concerning it, all my informants concurring in the conclusion that it is really an excellent formula. My able friend Professor GILSON has tried it with a variety of objects, and has been good enough to give me the following details. The nuclear staining is very sharp and good, the chromatic elements being coloured of a somewhat slaty blue. Cytoplasm is of a more or less violet or more or less orange red, and caryoplasm is of the same colour as the cytoplasm, but of a lighter tone. Cell-membranes, nuclear membranes, achromatic fibrillar structures, the Nebenkern when present—in a word, all the denser protoplasmic structures, are stained of the same colour as the hyaloplasm, but darker. The stain

possesses the usual qualities of mixtures of Methyl Green and Acid Fuchsin, the Orange apparently coming into play only in special cases. The stain works well, and may be put into the hands of beginners. It is on the whole an excellent formula, and will give valuable results in many cases, but possesses no special magic, and is not likely to supersede those amongst other staining methods that extended experience has found to be valuable. It may not be amiss here to repeat the warning that there neither is nor can be such a thing as a universal reagent in histology—"nouvel objet, nouvelle méthode!"

Gilson adds that for cytological purposes the proportions of the ingredients should be varied according to circumstances. For cells whose nucleïn is less chromophilous than the average, the proportion of methyl green should be increased, and in the opposite case the proportion of acid fuchsin should be increased.

For the special purpose of obtaining a minutely precise stain of attraction spheres and other cytoplasmic elements, HEIDENHAIN recommends that the mixture be acidified. For the somewhat minute details of the process and of the results, see § 639 in "Cytological Methods" in Part II.

The stain will work with chrome-osmium mixtures, but I believe does not give so fine a coloration with them as with sublimate.

Dr. LINDSAY JOHNSON writes me, "For nerve tissues, brain and cord, retina and cochlea, &c., nothing in my opinion can beat the Ehrlich-Biondi stain to which about one third of twenty per cent. (saturated) solution of nigrosin is added" (see the paragraphs on "Retina" in Part II). Dr. Johnson also points out that it has the advantage of giving a stain whose colours are photographically correct; he says "it is *the stain par excellence* for photography, except where there are fibrous or cartilaginous tissues, which it stains red."

I cannot say to what extent the stain is permanent. Heidenhain says that the preparations made according to his acid method mentioned above are absolutely permanent.

260. Methyl Green and Eosin (CALBERLA, *Morph. Jahrb.*, iii, 1877, Heft 3, p. 625).—Mix 1 part of eosin with 60 parts of methyl green, and dissolve the mixture in warm 30 per cent. alcohol.

Sections stain in this solution in five or ten minutes; they should be quickly washed in successive alcohols, and mounted in balsam or glycerin.

261. Methyl Green and Eosin (List, *Zeit. f. wiss. Mik.*, ii, 1885, p. 147).—Stain for a few minutes in a mixture of three parts of absolute alcohol with one part of aqueous solution of eosin (0·5 per cent.), wash, and stain for five minutes in 0·5 per cent. aqueous solution of methyl green. Wash, dehydrate, clear, and mount in balsam. The preparations do not keep well in glycerin.

The method may be varied (l. c., p. 150) by diluting the methyl green solution with fifty volumes of water, and staining for twenty-four hours.

It may also be varied by diluting the original methyl green solution with three volumes of absolute alcohol.

The preparations should not be left in the alcohol used for dehydrating after the colour of the eosin has begun to reappear.

261a. Methyl Green and Eosin (Rhumbler, *Zool. Anzeig.*, No. 411, 1893, p. 47).—Make a mixture of equal parts of 1 per cent. aqueous methyl green solution, of a solution of 0·8 grm. of eosin in 50 per cent. alcohol (quantity not stated), and of absolute alcohol. Stain (sections or small pieces of material fixed with picro-sulphuric acid or alcohol) for half an hour; wash out first with water, then with successive alcohols, for not more than a quarter of an hour, clear and mount. The novelty contained in this paper consists in the somewhat astonishing statement that the stain serves to differentiate living substance from dead or inorganic substance. "All substance that was living at the time of fixation being stained of a sharp red, all dead substance or inorganic substance of a sharp green."

262. Methyl Green and Bismarck Brown (List, *Zeit. f. wiss. Mik.*, ii, 1885, p. 145).—Stain for a few minutes in Weigert's Bismarck brown (§ 110), wash, and stain in 0·5 per cent. aqueous solution of methyl green. Clear with bergamot oil or xylol, and mount in balsam.

Or, dilute the Bismarck brown for staining with three volumes of absolute alcohol, wash out with strong alcohol, and stain for a few minutes in the methyl green solution diluted with three volumes of absolute alcohol.

Or, stain for twenty-four hours in the Bismarck brown solution diluted with fifty volumes of water, and then for twenty-four hours in the methyl green solution diluted with fifty volumes of water.

263. Methyl Green and Nitrate of Rosanilin (List, l. c.).—Stain for a few minutes in 0·5 per cent. aqueous solution of methyl green, wash, and stain (l. c., iii, 1886, p. 393) for ten to fifteen minutes in 0·0001 per cent. aqueous solution of nitrate of rosanilin, wash out (rapidly) with absolnte alcohol, and clear.

264. Anilin Green and Bismarck Brown (List, *l. c.*).—To be used in the same way as methyl green and Bismarck brown *supra* (§ 262), and giving much the same results.

265. Anilin Green and Eosin (Schiefferdecker, *Arch. f. Mik. Anat.*, xv, 1878, p. 30).—Stain in a solution made by adding a few drops of aqueous solution of eosin to a watch-glassful of alcohol for from half an hour to several hours, wash with water, and stain for a few minutes in 1 per cent.

aqueous solution of anilin green. Wash with water, wash out with alcohol, and clear with clove oil.

This combination possesses a rich selectivity, especially for connective and glandular tissues.

LIST (l. c., § 262) recommends this combination, which he employs in the following manner:—Stain for a quarter of an hour in the eosin solution there quoted, rinse with alcohol, and stain for a quarter of an hour in 0·5 per cent. aqueous solution of anilin green diluted with three volumes of absolute alcohol. Wash out with absolute alcohol and (as soon as the colour of the eosin begins to reappear) clear with bergamot oil or xylol.

266. Dahlia and Eosin (SCHIEFFERDECKER, l. c.).—Proceed as for anilin green and eosin (§ 265), using a 1 per cent. aqueous solution of dahlia.

267. Methyl Violet and Eosin (SCHIEFFERDECKER, l. c.).—Proceed as before, using a 1 per cent. aqueous solution of methyl violet.

268. Baumgarten's Fuchsin and Methylen Blue (*Zeit. f. wiss. Mik.*, i, 1884, p. 415).—Stain sections (of chromic objects) for twenty-four hours in a stain made by adding 8 to 10 drops of concentrated alcoholic solution of fuchsin to a watch-glassful of water. Rinse with alcohol, and stain for four or five minutes in concentrated aqueous solution of methylen blue, wash out with alcohol for five to ten minutes, and clear with clove oil. Nuclei red, tissues blue, the fuchsin having been driven out of the tissues by the methylen blue, a result which is *not* attained by washing with alcohol alone, either pure or acidified.

269. Safranin and Indigo-Carmine (KOSSINSKI, *Zeit. f. wiss. Mik.*, vi, 1, 1880, p. 61).—Stain sections ten to twenty minutes in saturated aqueous solution of indigo-carmine, wash with water and with alcohol, and stain with safranin (0·5 per cent. in dilute alcohol), dehydrate and mount.

Safranin and Nigrosin is a combination also recommended by the same author. Stain for three to five minutes in 0·1 per cent. aqueous solution of nigrosin, and proceed as before.

Other Combinations.

270. Baumgarten's Triple Stain (*Bull. Soc. Belg. Mic.*, 7, 1887, and 14, 1888, p. 146; *Journ. de Microgr.*, 1888, p. 415; *Journ. Roy. Mic. Soc.*, 1887, p. 676, and 1889, p. 149). **Garbini's Safranin and Anilin Blue** (*Zool. Anz.*, 1886, p. 26; and *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 170). (Both these are much too complicated to be generally recommendable.) **Iodine Green and Eosin** (STIRLING, *Journ. Anat. and Physiol.*, xv, 1881, p. 354). **Rose Bengale and Iodine Green** (GRIESBACH, *Zool. Anzeig.*, 135, 1883, p. 172). **Roseïn and Anilin Blue, Roseïn and Anilin Green, Anilin Violet and Anilin Blue, Anilin Violet and Anilin Green** (see H. GIBBES, *Journ. Roy. Mic. Soc.*, 1880, p. 391). **Metallic Stains and Anilin Colours.**—Most of the coal-tar colours above quoted may be employed after a metallic impregnation. The combination of safranin with gold chloride, first recommended by Pfitzner, is a classical stain. Eosin may usefully be made to follow nitrate of silver impregnation. And other combinations may be found useful on occasion.

CHAPTER XIV.

IMBEDDING METHODS—INTRODUCTION.

271. A Word on Microtomes.—It is no part of the purpose of this work to discuss instruments, yet a word on this subject may be helpful to the student. The freezing microtome so generally employed in England is less than any other form adapted to the wants of the zoologist. Very thin sections can be obtained by it more readily than with any other microtome, but they are of little use when obtained. The relations of the parts of the organs are deranged by the freezing and by the thawing, and the aqueous nature of the process prevents it from being readily applicable to the mounting of *series* of sections. The microtome of the zoologist, therefore (I am not writing merely for pathologists or for dilettanti), must be an *imbedding* microtome. The two most important points to be attended to in the choice of such a microtome are the *object-holder* and the *knife motion*. The object-holder should *never*, as in some forms, be a well in which *the imbedded object* is raised by a screw; the principle of construction should always be that the *object-holder be raised* in its entirety by the screw, *not* the object alone. The *knife motion* should always be mechanical, the knife being guided by a mechanism giving the required precision of stroke. And the *object-holder* should be fitted with mechanical motions allowing of the orientation of the object in all three directions of space. This is a most important point; such an object-holder is absolutely necessary in many delicate morphological researches.

Amongst microtomes fulfilling these conditions various forms will be found almost equally convenient. Zeiss makes a good one; Schanze, of Leipzig, makes a good one; Reichert, of Vienna, makes a good one. All these are relatively cheap, and, being at the same time perfectly efficient for easy work, may be recommended. Amongst more precise instruments

the first place in order of date belongs to *the* THOMA *sliding microtome.* This is made in several sizes by R. Jung, Mechaniker in Heidelberg. For zoological and general histological work I recommend the *medium size* (No. 2*a* or 4), with the newest Naples object-holder and newest form of knife and knife-holder.

This instrument is described in *Journ. Roy. Mic. Soc.* (N.S.), vol. iii, p. 298; the new Naples object-holder (which I consider *essential* for the zoologist) is described and figured p. 915.

The BECKER *microtome* is in many respects an improvement on the Thoma model. It is essentially on the same principle, but possesses a mechanical arrangement for moving the knife-carrier; that is, the knife-carrier is not only *guided* by a mechanical arrangement, as in the Thoma model, but is *put in motion* by mechanism. This, I think, is certainly an advantage. Another improvement is that the slides are made of glass instead of metal; this allows one to dispense with the use of oil to the slides, which in the Thoma model gives rise to inequality in the thickness of sections. A minor point is that the instrument is somewhat cheaper than the Thoma form. It is made by Aug. Becker, Göttingen. Descriptions of two forms (Spengel and Schiefferdecker) will be found in *Journ. Roy. Mic. Soc.*, 1886, pp. 884 and 1084. The Naples object-holder can be fitted to the Becker microtome.

The instruments above described are "all-round" microtomes; by which is meant that they may be used either with a square-set knife or an obliquely-set knife, and will cut either celloidin sections or frozen preparations (if a freezing apparatus be added to them) just as well as paraffin sections. They will not, according to my experience, cut series of paraffin sections with quite the same infallible regularity, certainly not with the same rapidity as the instruments next to be mentioned. But they give results of almost the highest attainable quality, and in view of their adaptability to celloidin or other semi-soft preparations, I think that one of them, the Becker by preference, should be the instrument chosen by the worker who desires not to be entirely confined to the paraffin method, and who cannot conveniently possess more than one microtome.

The beautiful *Cambridge rocking microtome* (furnished by the Cambridge Scientific Instrument Company, St. Tibb's

Row, Cambridge, price £5, or by Messrs. Swift and Son, or by Jung) is only adapted for cutting paraffin sections (Mr. Swift has shown me an arrangement for inclining the knife so as to give it the position required for cutting celloidin; but I feel pretty sure that this will prove a failure in practice). This instrument is extremely simple and extremely rapid, and, what is more important, cuts more level series of sections than any other microtome I am personally acquainted with. It should be fitted with the improved moveable object-holder of Henneguy and Vignal (*Compt. Rend. Soc. Biol.*, 1885, p. 647), or some equivalent arrangement allowing the precise orientation of the object. (This, as well as the entire instrument, is manufactured in France by Dumaige, 9, Rue de la Bucherie, Paris, or Messrs. Swift on request will furnish such an arrangement, or it may be obtained, with or without the entire instrument, from Jung, of Heidelberg.)

It has been objected to this instrument by Schiefferdecker (see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 171, a description and criticism of the instrument as made by Jung) that it does not cut plane sections, but sections having the form of segments of a cylinder. This is true; but it does not therefore follow, as Schiefferdecker concludes, that the instrument is inapplicable to many morphological purposes, and especially to embryological research. In practice, the slight deviation of the sections from a plane figure is found to be quite inappreciable, and therefore unimportant. The chief defect of the instrument from the point of view of the morphologist is that it will not cut through a sufficiently lengthy series of sections without having the object-holder readjusted. Now readjustment of the object-holder is a ticklish piece of work, even with the precise and well-situated Naples object-holder mentioned above in connection with the Thoma microtome; much more is this the case with the relatively imperfect and ill-situated object-holders supplied with the Cambridge microtome. An ideal microtome ought to be able to section at least a centimetre of tissue without readjustment; the Cambridge will only manage about a millimetre.

For object-holders see the price list of JUNG; also a paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 165.

Lastly, I would call attention to the MINOT microtome made by E. Zimmermann, Mechaniker, 37, Halle'sche Strasse,

Leipzig-Gohlis. A description and figures of this instrument will be found in *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 176. It is worked on the sewing-machine principle : the knife is fixed as in the Cambridge instrument, and the object is made to impinge on it by means of a rotary motion given to a wheel by the hand, and converted by a crank and slide lever into a vertical one given to a slide carrying the object. This microtome cuts with very great rapidity, and those who have worked with it speak very highly of it. I have not tried it myself, but make no doubt the recommendation is justified, though, looking to the construction of the slide, I doubt whether it will afford sections quite so true as those given by a carefully handled Becker. The object-holder does not appear to be so scientifically constructed as the Naples one. Like the Cambridge instrument, this microtome is only adapted for paraffin work, and for this reason and the others stated above I do not feel satisfied that it should be preferred to the Becker or Thoma by those who have to be content with a single instrument.

272. Imbedding Methods.—The beautiful processes known as Imbedding Methods are employed for a twofold end. Firstly, they enable us to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed, the included object may be cut into sufficiently thin slices without distortion. Secondly, they enable us to fill out with the imbedding mass the natural cavities of the object, so that their lining membranes or other structures contained in them may be duly cut *in situ ;* and, further, they enable us to surround with the supporting mass not only each individual organ or part of any organ that may be present in the interior of the object, but each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the details of structure will precisely retain their natural relations of position. Such a process of imbedding is at the same time practically a process of hardening in so far as it gives to tissues a degree of firmness that

could otherwise only be obtained by the employment of chemical processes such as prolonged treatment with chromic acid and the like.

These ends are usually attained in one of two ways. Either the object to be imbedded is saturated by soaking with some material that is liquid while warm and solid when cold, which is the principle of the processes here called *Fusion Imbedding Methods;* or the object is saturated with some substance which whilst in solution is sufficiently fluid to penetrate the object to be imbedded, whilst at the same time, after the evaporation or removal by other means of its solvent, it acquires and imparts to the imbedded object sufficient firmness for the purpose of cutting. The collodion process sufficiently exemplifies this principle. If a piece of soft tissue be dehydrated, and soaked first in ether and then in collodion, and if the ether contained in the collodion be allowed slowly to evaporate, the tissue and surrounding mass of collodion will acquire a consistency such as to admit of thin sections being cut from them. The methods founded on this principle are here called *Evaporation Imbedding Methods.*

The now antiquated egg-emulsion process, in which a mass that is liquid whilst cold is coagulated by heat, or other agency, belongs to a third class, which may be described as *Coagulation Methods.*

In any of these cases the material used for imbedding is technically termed an "imbedding mass"—*Einbettungsmasse* —*masse d'inclusion.* Imbedding methods are spoken of by French writers as *méthodes d'inclusion,* or *méthodes d'enrobage.*

273. Imbedding Manipulations.—Before proceeding to describe in detail the more important imbedding methods, it is necessary to give an account of the manipulations of the process of imbedding in general.

Imbedding in a melted mass such as paraffin is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it; at the moment when the mass has cooled so far as to have a consistency that will not allow the object to sink to the bottom, the object is placed on its surface, and more melted mass poured on until the object is enclosed. Or the paper

tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case when the mass is cold, the paper is removed from it before cutting.

To make **paper trays**, proceed as follows. Take a piece of stout paper or thin cardboard, of the shape of the annexed figure (Fig. 1); thin (foreign) post-cards do very well indeed. Fold it along the lines *a a'* and *b b'*, then along *c c'* and *d d'*, taking care to fold always the same way. Then make the folds *A A'*, *B B'*, *C C'*, *D D'*, still folding the same way. To do this you apply *A c* against *A a*, and pinch out the line *A A'*, and so on for the remaining angles. This done, you have an imperfect tray with dogs' ears at the angles. To finish it, turn the dogs' ears round against the ends of the box, turn down outside the projecting flaps that remain, and pinch them down. A well-made post-card tray will last through several imbeddings, and will generally work better after having been used than when new.

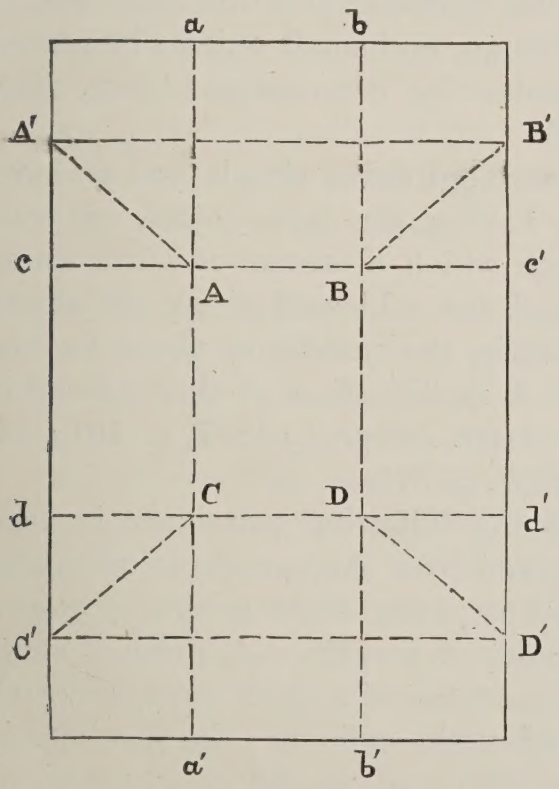

Fig. 1.

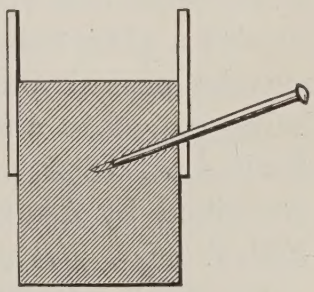

Fig. 2.

To make **paper thimbles**, take a good cork, twist a strip of

paper several times round it so as to make a projecting collar, and stick a pin through the bottom of the paper into the cork. For work with fluid masses, such as celloidin, the cork may be leaded at the bottom to prevent it from floating when the whole is thrown into spirit or other liquid for hardening (Fig. 2).

Leuckhart's **Imbedding Boxes** are made of two pieces of type-metal (Fig. 3). Each of these pieces has the form of a carpenter's "square" with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerin and gently warmed. The area of the box will evidently vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. In such a box the paraffin may be kept in a liquid state by warming now and then over a spirit lamp, and small objects be placed in any desired position under the microscope (*Journ. Roy. Mic. Soc.* [N.S.], ii, p. 880).

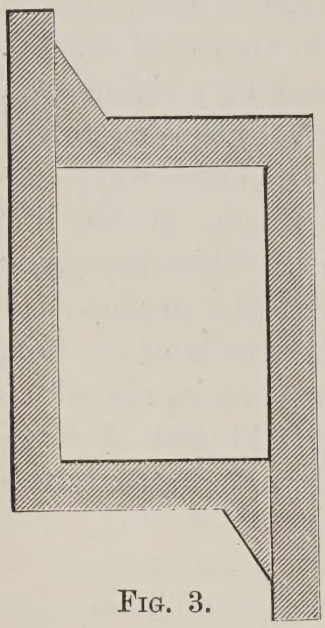

Fig. 3.

Selenka has described and figured a simple but perhaps more efficacious apparatus having the same object. It consists of a glass tube, through which a stream of warm water may be passed and changed for cold as desired, the object being placed in a depression in the middle of the tube (see *Zool. Anz.*, 1885, p. 419). A modification of this method is described by Andrews in *Amer. Natural.*, 1887, p. 101 ; cf. *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 375.

For **small paraffin objects** the following procedure is very useful. The object is removed from the paraffin solution, the superfluous fluid is removed by means of blotting-paper, and the object placed on a cylinder of paraffin. A piece of stout iron wire is now heated in the flame of a spirit lamp, aud with it a hole is melted in the end of the cylinder ; the specimen is pushed into the melted paraffin, and placed in any desired position. The advantages of the method lie in the quickness and certainty with which it can be performed.

I strongly recommend the reader not to neglect this simple method, which is capable of sometimes rendering services which no other method can. Those who have to do work with objects so small that their position can only be made out with the aid of a powerful lens ought to know how to arrange an object with a heated needle under a dissecting microscope, or on the object-carrier of the microtome.

[In the first edition this procedure was attributed to KINGSLEY. It appears to have been first published by BORN, see "Die Plattenmodellirmethode," in *Arch. f. mik. Anat.*, 1883, p. 591.]

There remains **the watch-glass method**. Melt paraffin in a watch-glass, and throw the object into it; or place the object in the watch-glass, add solid paraffin, and heat. After the mass has hardened, cut out a block containing the object (this is of course applicable to other masses, such as celloidin). If paraffin be used you may, instead of cutting out a block, turn out the whole mass of paraffin by simply warming rapidly the bottom of the glass. To facilitate the removal of the mass some persons lubricate the watch-glass before pouring in the mass. To do this a drop of glycerin should be smeared over it and wiped off with a cloth until hardly a trace of it remains.

As regards the merits of the watch-glass process, I wish to say that, as regards small objects at all events, I consider it the very best process of any.

274. Choice of a Method.—Amongst the very various methods of imbedding that have been proposed two are pre-eminently important—the paraffin method for small objects, and the celloidin or collodion method for large objects.

The subject of the respective merits of paraffin and celloidin still affords matter for discussion to some persons. The case, however, seems to be a very simple one. Celloidin does not afford by a long way the thinnest sections that are obtainable with small objects. For such objects it is, therefore, not equal to the demands made by modern minute anatomy, and paraffin must be taken. On the other hand, paraffin (as at present employed) will only cut very thin sections with small objects; with objects of 7 millimetres diameter you cannot get with paraffin thinner sections than you can with celloidin; and if

you try to cut in paraffin objects of somewhat greater size, 10 mm. and upwards, it will probably happen that you will not get perfect sections at all, blocks of paraffin of this size having a tendency to split under the impact of the knife ; so that for large objects celloidin gives better results.

I have not been able to satisfy myself that the preservation of the tissues is better in celloidin sections than in paraffin sections; so that—convenience apart—the case remains as above stated,—paraffin for small sections, celloidin for large ones.

To this must be added aqueous masses, such as gum or gelatin, for very special cases.

It will be the purpose of the next chapter to describe the paraffin method, and to mention some other masses that can be employed in a similar manner, the celloidin method and the other methods that remain to be described being treated of in Chap. XVI.

CHAPTER XV.

IMBEDDING METHODS—PARAFFIN AND OTHER FUSION MASSES.

275. Penetration or Clearing.—The first stage of the paraffin method consists in the penetration or infiltration of the object by some substance which is a solvent of paraffin. The process may be called a clearing process, since the chief substances used for infiltration are also "clearing" agents.

The process of penetration or clearing should be carefully performed with well-dehydrated objects in the manner described in a former chapter.

Penetration liquids being merely liquids that are, on the one hand, miscible with alcohol, and on the other hand good solvents of paraffin, are as numerous as could be wished. Amongst them may be mentioned essence of turpentine, clove oil, bergamot oil, creasote, benzol, xylol, toluol, naphtha, oil of cedar wood, chloroform, and anilin oil.

Turpentine penetrates well and mixes readily with paraffin. I do not, however, recommend it, because in my experience it is of all others the clearing agent that is the most hurtful to delicate structures.

Clove oil penetrates well, and preserves delicate structures well; but it mixes very slowly with paraffin, and quickly renders tissues brittle.

Benzin has been recommended by Brass (*Zeit. f. wiss. Mik.*, ii, 1885, p. 301).

Toluol (or toluen) has been recommended by Holl (*Zool. Anz.*, 1885, p. 223).

Naphtha has been recommended by Webster (*Journ. Anat. and Physiol.*, xxv, 1891, p. 278). For large specimens it has the advantage of being very cheap. Dr. Webster writes me that a quality known as "Persian naphtha" is best for fine work, but the common pure naphtha is sufficient for ordinary work.

Xylol is said by Heidenhain (see *Zeit. f. wiss. Mik.*, ix, 2,

1892, p. 200) to be a cause of shrinkage in cells; he employs oil of bergamot.

Chloroform mixes well with paraffin, and after evaporation in a paraffin bath (in the manner described in the next paragraph) leaves behind a pure and very homogeneous paraffin, having but little tendency to crystallise. But it is deficient in penetrating power, so that it requires an excessive length of time for clearing objects of any size; and it must be very thoroughly got rid of by evaporation in the paraffin bath, or by successive baths of paraffin, as if the least trace of it remains in the paraffin used for cutting it will make it soft. Chloroform ought, therefore, to be reserved for small and easily penetrable objects.

Cedar-wood oil is, according to my continued experience, for the reasons stated by me in *Zool. Anz.*, 1885, p. 563, in general the best clearing agent for paraffin imbedding. It penetrates rapidly, preserves delicate structure better than any clearing agent known to me, does not make tissues brittle, even though they may be kept for weeks or months in it, and has the great advantage that if it be not entirely removed from the tissues in the paraffin bath it will not seriously impair the cutting consistency of the mass; indeed, I fancy it sometimes improves it by rendering it less brittle.

In some difficult cases anilin oil is indicated (see § 353).

276. The Paraffin Bath.—The objects having been duly "penetrated" or "cleared," the next step is to substitute melted paraffin for the penetrating or clearing medium.

Some authors lay great stress on the necessity of making the passage from the clearing agent to the paraffin as gradual as possible, by means of successive baths of mixtures of clearing agent and paraffin kept melted at a low temperature, say 35° C. With oil of cedar or toluol, at all events, this is not necessary. All that is necessary is to bring the objects into melted paraffin kept just at its melting-point, and keep them there till they are thoroughly saturated; the paraffin being changed once or twice for fresh only if the objects are sufficiently voluminous to have brought over with them a notable quantity of clearing agent.

The practice of giving successive baths first of soft and then of hard paraffin appears to me *entirely illusory*.

It is important to keep the paraffin *dry*—that is, protected from vapour of water during the bath.

It is still more important to keep it as nearly as possible at melting-point. If it be heated for some time to a point much over its normal melting-point, *the melting-point will rise*, and you will end by having a harder paraffin than you set out with. And as regards the preservation of tissues, of course the less they are heated the better.

The *duration of the bath* must, of course, vary according to the size and nature of the object. An embryo of the size of a pea ought to be thoroughly saturated after an hour's bath, or often less, if cedar oil has been used for clearing. In any case, the preparations should be *cooled* (see below, § 279) *as soon as saturated*. If left for many hours in a warm bath, as is sometimes done, delicate structures may be seriously injured. Indeed, the great point to be attended to in paraffin work of the finer order is to minimise the action of heat. It is therefore important both to employ a paraffin of the lowest melting-point that will give good sections (see below, §§ 280, 285), and to abbreviate the warm bath as much as possible.

If chloroform or other volatile agent be taken, choice may be made of two methods: either, as in Giesbrecht's method, the chloroform containing the objects is heated to the melting-point of the paraffin, and the paraffin gradually added, and the mass kept at the melting-point of the pure paraffin until all the chloroform is driven off; or, as in Bütschli's method, the objects are simply passed direct from chloroform into a solution of paraffin in chloroform, in which they remain until thoroughly impregnated (half to one hour), and which is then evaporated at the melting-point of the paraffin. Bütschli recommends a paraffin solution melting at 35°. (Such a solution is made of about equal parts of chloroform and paraffin of 50° melting-point.) Or, in the case of larger objects, instead of evaporating the chloroform (which is often a very long process, as the chloroform must be *completely* driven off, or the mass will remain too soft for cutting), Bütschli simply transfers them from the bath of paraffin solution to a bath of pure paraffin.

Giesbrecht's method (*Zool. Anz.*, 1881, p. 484), more fully stated, is as follows:

Objects to be imbedded are saturated with absolute alcohol,

and then brought into chloroform (to which a little sulphuric ether has been added if necessary, in order to prevent the objects from floating). As soon as the objects are saturated with the chloroform, the chloroform and objects are gradually warmed up to the melting-point of the paraffin employed, and during the warming small pieces of paraffin are by degrees added to the chloroform. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum.

277. Stoves and Water-baths.—It is important that the paraffin should not be exposed to a moist atmosphere whilst it is in the liquid state. If a water-bath be used for keeping it at the required temperature, provision should be made for protecting the paraffin from the steam of the heated water.

A very convenient apparatus for this purpose is that of Paul Mayer, which will be found described at p. 146 of *Journ. Roy. Mic. Soc.*, 1883. It may be procured from the Zoological Station at Naples (address—"Direzione della Stazione Zoologica, Napoli"), or from M. Paul Rousseau, 17, Rue Soufflot, Paris. See also *Amer. Natural.*, 1886, p. 910; and *Journ. Roy. Mic. Soc.*, 1887, p. 167.

Other similar forms of paraffin-heating apparatus are described in several places in the same journal, as also in *Zeit. f. wiss. Mik.*

But whenever the worker has gas at his disposition, it will be found infinitely preferable to employ a regulating stove or thermostat. I recommend the form described in Fol's *Lehrbuch*, p. 121. Other descriptions of similar apparatus will be found also in the above-named journals.

278. Imbedding in Vacuo.—There are objects which, on account of their consistency or their size, cannot be penetrated by paraffin in the ordinary way, even after hours or days in the bath. For such objects the method of imbedding in a vacuum renders the greatest service. It not only ensures complete penetration in a very short time—a few minutes—but it has the further advantage of preventing any falling in of the tissues such as may easily happen with objects possessing internal cavities if it be attempted to imbed them in the ordinary way.

The principle of this method is that the objects are put through the paraffin-bath *in vacuo*. In practice this may be realised by means of any arrangement that will allow of maintaining paraffin at the necessary temperature for keeping it fluid under a vacuum.

The apparatus of Hoffmann will be found described and figured at p. 230 of *Zool. Anz.*, 1884. In this arrangement the vacuum is produced by means of a pneumatic water aspiration pump, the vessel containing the paraffin being placed in a desiccator heated by a water-bath and furnished with a tube that brings it into communication with the suction apparatus.

This arrangement is very efficacious and very simple if the laboratory possesses a supply of water under sufficient pressure.

In order to obtain the requisite vacuum without the aid of water under pressure, a simple little apparatus has been designed by FRANCOTTE (*Bull. Soc. Belg. Micr.*, 1884, p. 45). In this the vacuum is produced by the condensation of steam.

FOL (*Lehrb.*, p. 121) employs the vacuum apparatus of Hoffmann, but simplifies the arrangement for containing the paraffin. The paraffin is contained in a stout test-tube furnished with a rubber stopper traversed by a tube that puts it into communication with the pump. The lower end of the test-tube dips into a water-bath. You pump out the air once or twice, wait a few minutes to make sure that no more bubbles rise, then let the air in, turn out the object with the paraffin (which by this time will have become abnormally hard), and re-imbed in fresh paraffin.

See also a paper by PRINGLE, in *Journ. Path. and Bacteriol.*, 1892, p. 117; or *Journ. Roy. Mic. Soc.*, 1892, p. 893.

279. Imbedding and Cooling.—As soon as the objects are thoroughly saturated with paraffin they should be imbedded by one of the methods given above (§ 273). If the watch-glass method be followed the paraffin-bath will naturally have been given in the watch-glass used for imbedding, and no special imbedding manipulation will be necessary. In any case the important point now to be attended to is that *the paraffin be cooled as rapidly as possible*. The object of this is to prevent crystallisation of the paraffin, which may happen if it be allowed to cool slowly, and to get as homogeneous a mass as possible.

Very small objects may be taken out of the paraffin with a needle or small spatula, and put to cool on a block of glass, then imbedded in position for cutting on a cone of paraffin by means of a heated needle in the manner described above (§ 273). In the use of the needle it should be noted that it is important *to melt as little paraffin as possible at one time*, in order that that which is melted may cool again as rapidly as possible.

If the watch-glass method be adopted, float the watch-glass with the paraffin and objects on to cold water. Do not let it sink till all the paraffin has solidified. When cool, cut out blocks containing the objects; do this with a *slightly* warmed scalpel.

If paper trays be taken, cool them on water, holding them above the surface with only the bottom immersed until all the paraffin has solidified, as if you let them go to the bottom at

once you will probably get cavities filled with water formed in your paraffin. Or you may put them to cool on a block of cold metal or stone.

SELENKA recommends cooling the mass by passing a stream of cold water through the imbedding tube described above (§ 273).

The objects having been mounted on the carrier of the microtome in position for cutting, pare the blocks square to the knife, and sufficiently close down to the objects, and go round them with a lens. If any bubbles or cavities or opaque spots be present, prick with a heated needle till all is smooth and homogeneous. Minutes spent in this way are well invested.

It is well to cut within a few hours of imbedding if the structures be at all delicate, as paraffin may continue to crystallise slowly to a certain extent even after rapid cooling. But this danger is very greatly diminished if the mass have been properly cooled.

280. Cutting and Section-stretching.—Paraffin sections are cut *dry*—that is, with a knife not moistened with alcohol or other liquid. By this means better sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl up on the blade of the knife. It is sometimes difficult by any means to unroll a thin section that has curled. To prevent sections from rolling, the following points should be attended to:

First and foremost, the paraffin *must not be too hard*, but must be taken of a melting-point suitable to the temperature of the laboratory (for the winter season the temperature of the laboratory being between 15° and 17° C., a paraffin melting at about 45° C. should be taken; for hot summer weather, laboratory at 22° C., a paraffin of 48° C. melting-point).

The exact degree of hardness necessary must be determined by experiment. If, after cutting has begun, the paraffin be found to be too hard, it may be softened by the following simple expedient (FOL, *Lehrbuch d. vergl. mikr. Anat.*, p. 123):—A lamp provided with a parabolic reflector is set up near the microtome in such a position that the heat-rays of the flame are thrown by the reflector on to the imbedded object. The

right temperature is obtained by adjusting the distance of the lamp.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice placed in the focus of a similar reflector.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

Secondly, the knife should be set square, for the oblique position produces rolling, and the more the knife is oblique the more do the sections roll.

Thirdly, it is better to cut ribbons than disconnected sections; ribbons of sections will often cut perfectly flat even when the same mass will only give rolled sections if cut disconnectedly.

Special masses having less tendency to roll than pure paraffin have been proposed. Thus a mass composed of four parts of hard paraffin and one of vaselin has been recommended. I recommend, however, that all such mixtures be avoided.

Mechanical means may be employed. The simplest of these and perhaps the best is as follows:

During the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or by a small spatula made by running a piece of paper on to the back of a scalpel. Or the section is held down by means of an instrument called a "section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.

See the descriptions of various forms of section-stretchers, *Zool. Anzeig.*, vol. vi, 1883, p. 100 (SCHULTZE); *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 (MAYER, ANDRES, and GIESBRECHT); *Arch. f. mik. Anat.*, xxiii, 1884, p. 537 (DECKER); *Bull. Soc. Belge Mic.*, x, 1883, p. 55 (FRANCOTTE); *The Microscope*, February, 1884 (GAGE and SMITH); WHITMAN'S *Meth. in Mic. Anat.*, 1885, p. 91; *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 218 (STRASSER); as well as *Journ. Roy. Mic. Soc.*, iii, pp. 450, 916, and other places.

Another plan is to allow the sections to roll, but to control

the rolling. To this end, the block of paraffin is pared to the shape of a wedge five or six times as long as broad, the object being contained in the head or broad part, and the edge turned towards the knife. The sections are allowed to roll and come off as coils, the section of the object lying in the outermost coil, which will be found to be a very open one—indeed, very nearly flat. Lay the coil on a slide with this end downwards, warm gently, and the part containing the object will unroll completely and lie quite flat.

The most efficacious plan for unrolling sections is perhaps the combined treatment with fluid and heat (GASKELL, *Quart. Journ. Mic. Sci.*, xxxi, 1890, p. 382; M. DUVAL, *Journ. de l'Anat. et de la Physiol.*, 1891, p. 26; HENNEGUY, *ibid.*, 1891, p. 398; GULLAND, *Journ. of Anat. and Physiol.*, 1891, p. 56; and others). The rolled sections are either floated on to the surface of warm water or warm alcohol contained in a watch-glass or suitable dish, which causes them to flatten out, and are then transferred to a slide to be mounted in any desired manner. Or the slide has a layer of water spread over it, the sections are laid on the water, and the slide is heated (to about 45° to 50° C.) until the sections flatten out, which happens in a few instants. The method can be made available for fixing series of sections to the slide; the further details necessary for the successful accomplishment of this are given in Chap. XVII, § 324.

Minute examination of paraffin sections sometimes reveals certain distortions and dislocations or even ruptures of delicate elements. I have often noticed that in certain regions of my sections all the karyokinetic figures are drawn up to one side (always the same side) of the nucleus, leaving the rest of the nucleus empty and vacuolar in appearance. The achromatic fibrils of the division spindle are frequently ruptured, and I have not rarely found isolated chromosomes lying far from the nucleus in the body of cells, or even outside the cells themselves. These phenomena have generally been ascribed to "shrinkage" caused by the action of the fixing agents or the processes of dehydration or imbedding. HEIDENHAIN (*Ueber Kern u. Protoplasma*, in *Festschr. Herrn Geheimr. A. v. Kölliker gewidm.*, 1892; see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 200) thinks that they are often caused by *excessive tilt* of the under surface of the microtome knife. If this be found

to be the case, the knife should be readjusted by means of a piece of cardboard placed in the jaws of the clamp. I would suggest that another cause of these defects is to be found in imperfection of the edge of the knife. If the knife be blunt, or have a rounded or curled edge caused by untrue honing or stropping, it will of course act in respect to minute structures as a plough rather than a cutting instrument, and thus produce the appearances described.

281. Chain or "Ribbon" Section-cutting.—If a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not unfrequently happens that they adhere to one another by the edges so as to form a chain which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. The following appear to me to be the factors necessary for the production of a chain.

Firstly, the paraffin must be of a *melting-point* having a certain relation to the temperature of the laboratory. *Small* sections can always be made to chain when cut from a good paraffin of 45° C. *melting-point in a room in which the thermometer stands at* 16° *to* 17° C. (The temperatures quoted apply to the case of rooms heated by an open fire, and probably would not apply to the case of rooms heated by closed stoves, such as are usual in Germany.) At 15° C. the paraffin will be found a trifle hard. At 22° C. the proper melting-point of the paraffin will probably be found at about 48° C., but my observations at these temperatures are less extended. Secondly, the *knife should be set square.* Thirdly, the block of paraffin should be pared down very close to the object, and should be cut so as to present a straight edge parallel to the knife edge; and the opposite edge should also be parallel to this. Fourthly, the sections ought to be *cut rapidly,* with the swiftest strokes that can be produced. For it is the sharp impact of the knife that slightly heats, and therefore slightly softens the near edge of the paraffin, and thus causes the sections to cohere. It is evident that this condition can only be conveniently realised by means of a sliding microtome; but it is by no means necessary to have recourse to special mechanical contrivances, as in Caldwell's automatic

microtome or Strasser's ribbon microtome. The Thoma microtome well flooded with oil is sufficient. But the automatic microtomes are certainly most advantageous for this purpose, and amongst them the Cambridge Rocking Microtome, and the Minot, may be quoted as giving admirable results.

Various plans, such as coating the edges of the paraffin with softer paraffin, or with Canada balsam, or the employment of specially prepared paraffin, have been recommended, with the idea that they help the sections to stick. None of these devices is necessary. For the prepared paraffin of Spee, Brass, and Fœttinger, see below, § 286.

282. Collodionisation of Sections.—Some objects are by nature so brittle that, notwithstanding all precautions taken in imbedding and previous preparation, they break or crumble before the knife, or furnish sections so friable that it is impossible to mount them in the ordinary way without some impairment of their integrity. Ova are frequently in this case. The remedy for this state of things consists in covering the exposed surface of the object just before cutting each section with a thin layer of collodion, which serves to hold together the loose parts of even the most fragile sections in a wonderfully efficacious way; and the same treatment applied to tissues which are not specially fragile will enable the operator to cut sections considerably thinner than can be obtained in the usual way. Bütschli has obtained in this manner sections of less than 1 μ in thickness.

The primitive form of the process was to place a drop of collodion on the free surface of each section just before cutting it. But this practice has two defects; the quantity of collodion employed sensibly softens the paraffin, and the thick layer of collodion when dry causes the sections to roll.

Mark (*Amer. Natural.*, 1885, p. 628; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 738) gives the following directions:

"Have ready a little very fluid collodion in a small bottle, through the cork of which passes a small camel-hair brush, which just dips into the collodion with its tip. The collodion should be of such a consistency that when applied in a thin layer to a surface of paraffin it dries in two or three seconds without leaving a shiny surface. Collodion of this consistency

does not produce a membrane on the paraffin in drying, and therefore has no tendency to cause sections to roll. It has further the advantage that it penetrates to a certain depth below the surface of the preparation, and fixes the deeper layers of it in their places. The collodion must be diluted with ether as soon as it begins to show signs of leaving a shiny surface on the paraffin.

"Take the brush out of the collodion, wipe it against the neck of the bottle, so as to have it merely moist with collodion, and quickly pass it over the free surface of the preparation. Care must be taken not to let the collodion touch the vertical surfaces of the paraffin, especially not the one which is turned towards the operator, as that will probably cause the section to become stuck to the edge or under surface of the knife. As soon as the collodion is dry, which ought to be in two or three seconds, cut the section, withdraw the knife, and pass the collodion brush over the newly exposed surface of the paraffin. Whilst this last layer of collodion is drying, take up the section from the knife and place it with the collodionised surface downwards on a slide prepared with fixative of Schaellibaum. Then cut the second section, and repeat the manipulations just described in the same order. A skilful operator can cut ribbons of sections, collodionising each section."

Henking (*Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 478) objects to the above process that the ether of the collodion softens the paraffin, and proposes a solution of *paraffin* in absolute alcohol. The solution is made by scraping paraffin into absolute alcohol.

For extremely brittle objects, such as ova of Phalangida, the same author recommends a thin (light yellow) solution of *shellac* in absolute alcohol.

Heider (*Embryonalentw. v. Hydrophilus*, 1889, p. 12; cf. *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 509) employs a solution made by mixing a solution of gum mastic in ether, of a syrupy consistency, with an equal volume of collodion, and diluting the mixture with ether until quite thin and liquid.

283. Clearing and Mounting.—The sections having been obtained are generally mounted on a slide in serial order by one of the methods described in the chapter on "Serial Section

Methods." All that now remains is to get rid of the paraffin and mount or stain as the case may be. The following solvents of paraffin have been recommended for freeing sections from the paraffin with which they are infiltrated :—Turpentine, warm turpentine, a mixture of 4 parts of essence of turpentine with 1 of kreasote, kreasote, a mixture of turpentine and oil of cloves, benzin, toluol, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling-point. Any of these may be used, but naphtha and xylol are probably in most respects the best. Toluol also works very well.

If the slide be warmed to the melting-point of the paraffin, a few seconds will suffice to remove the paraffin if the slide be plunged into a tube of naphtha or toluol. The sections may be mounted direct from the naphtha, or the slide may be brought into a tube of alcohol to remove the naphtha for staining.

284. Recapitulation of the Paraffin Method, as recommended to be practised.—Put into a small test-tube enough oil of cedar to cover your object. On to the oil pour carefully the same quantity of absolute alcohol. Take your (already dehydrated) object and put it carefully into the alcohol. Leave it until it has sunk to the bottom of the cedar oil. Then put it into paraffin kept at melting-point in a watch-glass. Let the paraffin be of the *very lowest melting-point* that will give sufficiently thin sections, and to this end work in a cool place, so as not to be obliged to go above 45° C. if possible (see also next section). After a time change the paraffin (*i.e.* put the object into a fresh watch-glass with clean paraffin) once, or twice if the object be at all large. As soon as the object is thoroughly soaked with paraffin float the watch-glass on cold water. When cool, cut out a block of paraffin containing the object, and fix it with a heated needle on a cone of paraffin already mounted on the object-carrier of the microtome. Pare it square, and as close down to the object as possible on all sides except the one turned towards the knife; this had better have a wall of a millimetre or two, or more, according to the size of the object, left standing. Set the knife square. Set the block square to the knife-edge. Cut sections in

chains or ribbons, collodionising them if necessary. Fix them in serial order on a slide according to one of the methods given in Chap. XVII. Warm, and remove the paraffin with naphtha. Stain or mount.

Paraffin Masses.

285. Pure Paraffin.—It is now pretty generally admitted that pure paraffin forms an imbedding mass greatly superior for ordinary work to any of the many fatty mixtures that used to be recommended. I have only to repeat here that *a paraffin melting at* 45° C. is that which in my experience gives the best results *so long as the temperature of the laboratory is between* 15° *and* 17° C.; whilst for a temperature of 22° C. a paraffin melting at 48° is required. If the temperature of your laboratory have risen much above 22° C. you had better give it up, for good section-cutting with paraffin under such conditions is next to impossible.

Paraffin of the melting-points named is easily found in commerce. Intermediate sorts may be made by mixing hard and soft paraffin. Two parts of paraffin melting at 50° with one of paraffin melting at 36° C. give a mass melting at 48° C.

Many workers of undoubted competence prefer masses somewhat harder than those recommended, viz. of melting-points varying between 50° and 55° C. for the normal temperature of the laboratory. Some authors still recommend masses melting at 60° C. or higher. I can only repeat that I am convinced that, *besides being most hurtful to tissues, such masses have no* raison d'être *whatever in temperate climates.*

Paraffin had better be obtained from Grübler, or one of the known dealers in microscopic reagents. Gaule recommends that the bluish transparent sorts be taken. I should say, transparent by all means, but, if possible, rosy rather than bluish. New paraffin is bluish; if kept long, which is well, it generally becomes rosy.

286. Prepared Paraffin (Pure).—Graf Spee (*Zeit. f. wiss. Mik.*, ii, 1885, p. 8) recommends the following preparation of commercial paraffin as giving a mass particularly favorable for ribbon-section cutting. Paraffin of about 50° C. melting-point is taken and heated in a porcelain capsule by means of

a spirit lamp. After a time disagreeable white vapours are given off, and the mass shrinks a little. This result is arrived at in from one to six hours, according to the quality of the paraffin. The mass then becomes brownish yellow, and after cooling shows an unctuous or soapy surface on being cut. The melting-point will be found to have risen several degrees. This mass may be obtained ready prepared from Grübler.

Brass (loc. cit., p. 300) recommends the use of paraffin that has been kept for some years, as such has less tendency to crystallise than new paraffin. In this I concur.

Foettinger recommends (*Arch. de Biol.*, vi, 1885, p. 124) a somewhat complicated treatment with caustic potash, in which I have no faith (it was tried by one of the writers of the *Traité des Meth. techn.*, during the preparation of that work).

287. Paraffin Mixtures and other Similar Masses.—Of these the only ones that I think can be recommended for a moment are the following:—Schulgin's paraffin with a little cerisin (this is evidently what Schulgin means by "ceresin"). Or, instead of cerisin, white wax (see *Zool. Anz.*, 1883, p. 21); or the mixture of Brass (*Zeit. f. wiss. Mik.*, ii, 1885, p. 301), who recommends four to six parts of white wax to 100 of paraffin. Sections may be cleared with benzin.

Cacao Butter, pure, is still used by some histologists. It melts somewhat under 35° C. The objects may be prepared by penetration with clove oil, which may also be used for removing the mass from the sections.

Bayberry Tallow (Myrtle Wax, Vegetable Wax, Japan Wax).—See *Journ. Roy. Mic. Soc.*, 1885, p. 735; *ibid.*, 1888, p. 151; Miller and Blackburn, *New York Med. Rec.*, 1885, p. 429; *Amer. Mon. Mic. Journ.*, 1887, p. 164; *Journ. Roy. Mic. Soc.*, 1887, p. 1048; Francotte, *Bull. Soc. Belge de Mic.*, 1887, p. 140; *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 230; *Journ. Roy. Mic. Soc.*, 1887, p. 681. Bayberry tallow has the, for some objects, valuable quality of working with alcohol as a solvent instead of such solvents as chloroform or benzol, which undoubtedly alter certain tissues more than alcohol. But for general work it has not succeeded in taking the place of paraffin.

Soap Masses.

288. Utility of Soap Masses.—Soap masses certainly have many good points. The solvent is alcohol; the mass is highly transparent, very penetrating, and a good mass cuts far better than even paraffin. The mass may be cut either dry or with

alcohol. As to the preservation of tissues, the mass is alkaline, which is against it; yet some workers still prefer soap to paraffin, and it has lately been recommended by so experienced a worker as Chun, for Siphonophora (certainly as delicate a class of objects as any that exist), on the ground of its producing less shrinkage than paraffin.

289. Transparent Soap (Pölzam, *Morph. Jahrb.*, iii, 1877, 3tes Heft, p. 558).—The following account is taken from Salensky's paper on the gemmation of Salpa (loc. cit.).

Take good white soap ("gewöhnliche Kernseife"), cut it up into thin slices, and put them to dry in the sun for some days until they become white. The slices are then to be rubbed up to a fine powder, which is mixed with spirit to the consistency of porridge. Now mix the porridge with alcohol and glycerin in such proportions that the whole shall contain, for every ten parts by weight of the soap, 22 parts of glycerin and 35 parts of alcohol (90 per cent.). Let the whole simmer until there is obtained a perfectly transparent, syrupy, somewhat yellow fluid.

The objects, previously dehydrated in alcohol, are imbedded in this mass in the usual manner.

The mass may be removed from the sections either by means of water or of very dilute alcohol.

290. Transparent Soap (Kadyi, *Zool. Anz.*, 37, 1879, vol. ii, p. 477).—Twenty-five grms. of shavings of stearate of soda soap (any stearate of soda soap will do, but the most to be recommended is the sort known in commerce as "weisse Wachskernseife") are heated in a retort with 100 c.c. of 96 per cent. alcohol over a water-bath until the whole is dissolved. Filter if necessary. If a drop of the solution be now poured into a watch-glass it will be seen that it almost immediately solidifies into a *white* mass. This is not what is wanted, and is a sign that the solution does not contain water enough. Small quantities of water are therefore added by degrees to the solution, and the effect tested from time to time by pouring a drop of the mixture into a watch-glass. The mass will be seen to become more and more pellucid until a point is reached at which it is almost perfectly transparent, with merely the slightest blue opalescence. The preparation of the mass is then complete.

It is not possible to state *a priori* the exact proportion of water that should be added, as this naturally depends on the amount of water already present in the sample of soap taken. In very many cases it will be found that for about 120 grms. of soap solution 5 to 10 grms. of water will be required.

It is necessary to be very cautious in adding the water, as if too much be taken the mass solidifies more slowly or not at all. The greatest amount of elasticity and consistency is possessed by the mass at the moment in which it contains exactly the minimum amount of water necessary to make it transparent.

The reasons for this process are explained as follows:—Stearate of soda soap is soluble in divers proportions in warm alcohol. On cooling, the solution either solidifies into a homogeneous and pellucid mass, or into a white granular mass; or, in certain cases, does not solidify at all. The result in each case depends on the proportion of water present in the solution. For instance, if 5 to 6 parts of a tolerably dry soap be dissolved in 100 parts of 96 per cent. alcohol, a solution is generally obtained that solidifies into a transparent mass. But such a mass is too soft, and its melting-point too low; it melts by the heat of the finger. If now, in order to get a harder mass, you add more soap, you will get a solution that solidifies on cooling into a *white granular* mass; and it is only after adding to it a *certain* (*small*) quantity of water that you will obtain a solution that solidifies on cooling into a *transparent* mass. If you add more water than is just absolutely necessary to this end the mass will have too high a melting-point, and will solidify more slowly; and if still more water be added the solution will not solidify for hours, or, indeed, not at all. The more soap you have in your alcoholic solution the more water *must* you add in order to get a transparent mass, and the more *may* you add without depriving the solution of the faculty of solidifying. Besides the mass prepared in the proportions given above, useful masses may be made for certain purposes with 10, 20, 30, 40 per cent., or more or less of soap in alcohol. Weisker has employed a mass composed of about equal parts by weight of soap and alcohol. Such a mass is transparent, but yellow and oily, and takes a long time to solidify. When cool it is very tough. It requires a considerable temperature to liquefy it, and has less penetrating power than the more alcoholic masses. It is, however, very suitable for hard, and especially for chitinous structures.

The mass recommended above boils at about 60° to 70° C. Objects should be imbedded in it in a watch-glass or in paper cases in the usual way. Whilst cutting, wet the knife and the mass with strong alcohol (one advantage of this method is that the knife remains perfectly clean). The sections are brought into 96 per cent. alcohol, which frees them from

the mass instantaneously if warmed, and after a time if left cold.

291. Other Soap Masses (Flemming, *Arch. f. mik. Anat.*, 1873, p. 123; Pfitzer, *Ber. deutsch. bot. Ges.*, 1887, p. lxv; *Journ. Roy. Mic. Soc.*, 1888, p. 316; Poli, *Journ. de Micr.*, xiii, 1889, p. 337; *Journ. Roy. Mic. Soc.*, 1889, p. 835).—90 per cent.; alcohol, 1 part; glycerin, 1 part; glycerin soap, 1 to 2 parts. Heat the glycerin and spirit on a water-bath to 60° or 70° C., and drop the soap into it in small pieces.

Gelatin Masses.

292. Gelatin Imbedding is a method that has the advantage of being applicable to tissues that have not been in the least degree dehydrated, and may render great service in the study of very watery objects.

The *modus operandi* is, on the whole, the same as for other fusion masses, with the difference that the objects are prepared by penetration with *water* instead of alcohol or a clearing agent. After the cooling of the mass it may sometimes be cut at once, but it is generally necessary to harden it. This may be done by treatment for a few minutes with absolute alcohol (Kaiser), or for a few days with 90 per cent. alcohol (Klebs) or chromic acid (Klebs), or it may be frozen (Sollas).

The mass is removed from the sections by means of warm water.

293. Klebs' Gelatin (Glycerin Jelly) (*Arch. f. mik. Anat.*, v, 1869, p. 165).—A concentrated solution of isinglass is mixed with half its volume of glycerin.

294. Kaiser's Gelatin (*Bot. Centralb.*, i, 1880, p. 25; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 504).—One part by weight of the finest French gelatin is left for about two hours in 6 parts by weight of water; 7 parts of glycerin are added, and for every 100 grms. of the mixture 1 grm. of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass which has been previously washed in water and laid whilst wet in the filter.

295. Gerlach's Gelatin (Gerlach, *Unters. a. d. Anat. Inst. Erlangen*, 1884; *Journ. Roy. Mic. Soc.*, 1885, p. 541).—Take

gelatin, 40 grms.; saturated solution of arsenious acid, 200 c.c.; glycerin, 120 c.c. Clarify with white of egg. The mass may be kept for years in a well-stoppered bottle. The objects to be prepared for imbedding by a bath of one third glycerin.

296. Brunotti's **Cold Gelatin Mass** (*Journ. de Botan.*, vi, 1892, p. 194; *Journ. Roy. Mic. Soc.*, 1892, p. 706).—Twenty grms. gelatin dissolved with heat in 200 c.c. distilled water, and 30 to 40 c.c. of glacial acetic acid with 1 grm. corrosive sublimate added after filtering. At the temperature of 15° C. the mass has the consistence of a thick syrup. Objects are prepared by soaking in some of the mass diluted with two to three vols. of water, then imbedded in the undiluted mass. The mass is then hardened in spirit or bichromate of potash, picric acid, or the like. No heat at all is required in this process.

CHAPTER XVI.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS.

297. Collodion, Celloidin, and Photoxylin.—The collodion method is due to DUVAL (*Journ. de l'Anat.*, 1879, p. 185). *Celloidin*, recommended later on by MERKEL and SCHIEFFERDECKER (*Arch. f. Anat. u. Phys.*, 1882, p. 200), is merely a patent collodion.

It is stated to be a preparation of pure pyroxylin, and is patented for Germany and England under the name of "Schering's Celloidin." It is manufactured by the *Chemische Fabrik auf Actien* (vorm. E. Schering), Berlin, N. Fenstrasse 11, 12. It may be obtained through the post by writing to SCHERING'S "Grüne Apotheke," Wittick and Benkendorf, Berlin, N. Chaussée-Strasse, No. 19, or from GRÜBLER, or the other dealers in histological reagents.

It is stated to be prepared with the purest pyroxylin, and to be always of a uniform composition. It is sent out in the form of tablets of a tough gelatinous consistency and slightly milky-white transparency. These tablets contain 20 per cent. of pure pyroxylin. Celloidin is entirely soluble, in all proportions, in ether and alcohol. It is free from acids. It is not detonant. If ignited it burns like paper; heated in a test-tube it carbonises without exploding.

In order to make a 2 per cent. collodion, take one tablet of celloidin (which contains 40 grms. of the dry pyroxylin), and such a quantity of alcohol and ether that the whole shall weigh 2000 grms. For a 3 per cent. collodion you take such a quantity of alcohol and ether that the whole shall weigh 1333 grms.; and for a 4 per cent. collodion such a quantity that the whole shall weigh 1000 grms. The relative portions of alcohol and ether may be taken according to discretion. To prepare a medicinal collodion according to the Prussian Pharmacopœia you take for each tablet 720 grains of ether and no alcohol, as the celloidin already contains the prescribed

proportion of alcohol. The tablets cost three marks (= three shillings) each. A single tablet would, I think, suffice for imbedding many hundreds of embryos.

There is a strife of opinion amongst authorities as to the relative merits of celloidin and common collodion. DUVAL, in a restatement of the method (*Journ. de Micr.*, 1888, p. 197), teaches that celloidin has no real advantage over common collodion, whilst the latter has the advantage of being more transparent. SCHIEFFERDECKER, also restating the method, declares that celloidin has "many points of superiority" (*Zeit. f. wiss. Mik.*, v, 4, 1888, p. 504). On carefully reading Schiefferdecker's paper, however, it appears that the only reason alleged in support of this superiority is that it is more "convenient" to make a thick mass by making a strong solution of celloidin than by allowing common collodion to concentrate by evaporation.

Personally I incline to Duval's point of view. The superiority of celloidin, if it exists, is mainly of the nature of a mere matter of convenience. Otherwise there is but little to choose between the two, and therefore in this work the terms collodion and celloidin are used indifferently.

Photoxylin (KRYSINSKY, VIRCHOW'S *Archiv*, cviii, 1887, p. 217; BUSSE, *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 47) is a dry substance, of the aspect of cotton wool, and chemically nearly related to celloidin. It can be obtained either from SCHERING or GRÜBLER. It gives a clear solution in a mixture of equal parts of ether and absolute alcohol, and should be used in exactly the same way as celloidin. It has the very great advantage of affording a mass which after hardening in 85 per cent. alcohol remains perfectly *transparent*.

298. Preparation of Objects.—The objects must first be thoroughly dehydrated with absolute alcohol. They are then soaked till thoroughly penetrated in ether, or, which is better, in a mixture of ether and absolute alcohol. DUVAL (l. c.) takes for this purpose a mixture of ten parts of ether to one of alcohol; SCHIEFFERDECKER (and the majority of workers) a mixture of equal parts of ether and alcohol; TUBBY (in *Nature*, November 17th, 1892, p. 51) advises a mixture of four parts of ether and one of alcohol. But the point is one of no great importance.

This stage may be omitted if the objects are of a sufficiently permeable nature, and they may be brought direct from alcohol into the collodion bath.

299. The Collodion Bath.—The next step is to get the objects penetrated with thick collodion. The secret of success here is to penetrate them first with thin solutions, then with the definitive thick one. (A thin solution may be taken to mean one containing from 4 to 6 per cent. of dry celloidin; a thick solution one containing 10 to 12 per cent.)

If collodion be taken the thin solution may be made by diluting it with ether. If photoxylin or celloidin be taken the solutions are made in a mixture of ether and absolute alcohol in equal parts. Apáthy recommends that celloidin be allowed to dry in the air until it becomes yellow, transparent, and of a horny consistency, and that it be then dissolved in the alcohol and ether (sulphuric, free from acid). The solutions thus prepared are free from the excess of water that is present in the undried celloidin, and give after hardening a mass that is more transparent and of a better consistency for cutting (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 164).

Busse (op. cit., ix, 1, 1892, p. 47) gives the following proportions for the successive baths:—No. 1, 10 parts by weight of photoxylin or perfectly dried celloidin to 150 parts of the ether and alcohol mixture; No. 2, 10 parts of photoxylin or celloidin to 105 of the mixture; No. 3, 10 parts to 80 of the mixture (already used solution may be employed for the first bath).

The objects ought to remain in the first bath until very thoroughly penetrated;—days, even for small objects,—weeks or months for large ones (human embryos of from six to twelve weeks, for instance). If the object contain cavities, these should be opened to ensure their being filled by the mass.

When the object is duly penetrated by the thin solution, or solutions if more than one have been employed, it should be brought into the thickest one. Schiefferdecker (l. c.) recommends that this be done by allowing the thin solution to concentrate slowly (the stopper of the containing vessel being raised, for instance, by means of a piece of paper placed under it), and making up the loss from evaporation with thick solution.

300. Imbedding.—At this stage, if it has not been done before, the objects must be imbedded—that is, arranged in position in the receptacle in which they are to be hardened. For the usual manipulations see § 273. I recommend the paper thimbles or cylindrical trays, Fig. 2, as being very convenient for collodion imbedding. The bottoms, however, should be made of soft wood in preference to cork; cork is elastic, and bends in the object-holder of the microtome, deforming the mass and object. The box should be prepared for the reception of the object by pouring into it a drop of collodion, which is allowed to dry. The object of this is to prevent bubbles coming up through the wood or cork and lodging in the mass.

Objects may also be imbedded on a piece of pith or leather, which should also be prepared with a layer of dry collodion.

Watch-glasses, square porcelain water-colour moulds, and the like, also make convenient imbedding receptacles. Care should be taken to have them perfectly *dry*. Any of these receptacles or supports may be set with the mass under a glass shade, allowing of just enough communication with the air to set up a slow evaporation. Or porcelain moulds or small dishes may be covered with a lightly fitting cover.

If it be desired to mark the position of the object in the mass in order to facilitate the subsequent orientation of it on the object-holder of the microtome, recourse may be had to the method described by EYCLESHEIMER in *Amer. Nat.*, xxvi, 1892, p. 354 (see also *Journ. Roy. Mic. Soc.*, 1892, p. 563). The object is imbedded in one of the metal boxes described in § 273. The box has its ends and sides perforated at regular intervals by small opposite holes. Silk threads are passed through these holes from side to side, stretched, and kept tight by sticking them to the sides of the box by means of a drop of celloidin, leaving a length of a couple of inches hanging loose. The loose ends are soaked in thin celloidin solution with which lampblack has been mixed. The object is arranged in position on the framework formed by the taut threads in the box, the mass is poured in, and the whole is hardened. After hardening, the celloidin holding the ends of the threads is dissolved by means of a drop of ether, and the lampblacked ends are pulled through the box. This leaves adhering to the bottom of the mass a series of black lines which form useful orientation points.

301. Hardening.—This is logically the next step, but as a matter of fact is frequently begun before. For the different processes of the collodion method so run into one another that it is difficult to assign natural lines of demarcation between them.

The objects being imbedded, and in the stage at which we left them at the end of § 299, the treatment should be as follows :—As soon as the added thick collodion (of which only just enough to cover the object should have been taken) has so far sunk down that the object begins to lie dry, fresh thick solution is added, and the whole is left as before. Provision should be made for slow evaporation, either in one of the ways above indicated, or, which is perhaps better, by setting the objects under an *hermetically* fitting bell-jar, which is lifted for a few seconds only once or twice a day. I have sometimes found it advantageous to set the objects under a bell-jar together with a dish containing alcohol, so that the evaporation is gone through in an atmosphere of alcohol. This is especially indicated for very large objects.

When the mass has attained a consistency such that the ball of a finger no longer leaves an impress on it, it should be scooped out of the dish or mould, or have the paper removed if it has been imbedded in paper, and be submitted to the next stage of the hardening process. (If the mass is found to be not quite hard enough to come away safely it should be put for a day or two into weak alcohol, 30 to 70 per cent.)

Several methods are available for the definitive hardening process. One of these is the *chloroform* method, due to Vialanes (*Rech. sur l'Hist. et le Dév. des Insectes*, 1883, p. 129). I recommend this method for *small* objects, because I find it more certain and more rapid than the alcohol method, and preferable on account of a superior consistency it gives to the mass. (Schiefferdecker does not find this, v. *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 506.) For *large* objects the method is inferior to the alcohol method, because the rapid hardening of the external layers is an obstacle to the diffusion necessary to the hardening of the inner layers.

The method consists in bringing the objects into *chloroform*. "Under the influence of this reagent the collodion coagulates into a mass having the consistence of wax, but having also an elasticity that renders it unbreakable, and having besides the precious quality of being admirably transparent, and possessing exactly the index of refraction of glass."

In some cases a few *hours'* immersion is sufficient to give the requisite consistence. In no case have my specimens required more than three days. But the length of time required

varies in a very inexplicable way, so that no rule can be given. The collodion frequently becomes opaque on being put into the chloroform, but regains its transparency after a time.

Small objects may be hardened by chloroform without preliminary hardening by evaporation. All that is necessary is to expose the mass to the air for a few seconds until a membrane has formed on it, and then bring it into chloroform. If the mass is in a test-tube this may be filled up with chloroform, and left for two or three days. By this time the collodion mass will be considerably hardened, and also somewhat shrunk, so that it can be shaken out of the tube. It is then brought into fresh chloroform in a larger vessel, where it remains for about six days, after which time it is generally ready for cutting.

Good chloroform is a necessity, as the reaction cannot be obtained with samples of chloroform that are not free from water.

The following *rapid method*, communicated to me by Prof. GILSON, has the advantage of being the most expeditious of any. The object is dehydrated, soaked in ether, and brought into a test-tube with collodion or thin celloidin solution. The tube is dipped into a bath of melted paraffin, and the collodion allowed to boil (which it does at a very low temperature) until it has become of a syrupy consistence. The mass is then turned out, mounted on a block of hardened celloidin, and the whole hardened in chloroform or in a mixture of chloroform and cedar oil for about an hour. It is then cleared in cedar oil (if hardened in pure chloroform; special clearing will not be necessary if it has been hardened in the mixture). It may now be fixed in the microtome and cut, using cedar oil to wet the knife, and cover the exposed surface of the object after each cut.

The more commonly employed hardening method is the *alcohol* method. The objects are thrown into alcohol and left there until they have attained the right consistency (one day to several weeks). The bottle or other vessel containing the alcohol *ought not to be tightly closed, but should be left at least partly open.*

The strength of the alcohol is a point on which the practice of different writers differs greatly. Some take very weak alcohol; so ROLLETT, one third alcohol (see ROLLETT's *Unters.*

üb. d. Bau d. quergestr. Muskelf., 1885; or *Zeit. f. wiss Mik.*, iii, 1, 1886, p. 93). Others take equal volumes of absolute alcohol and water. SCHIEFFERDECKER (*Zeit. f. wiss. Mik.*, v, 4, 1888) recommends alcohol of 50 to 60 per cent.; THOMA, alcohol of 82 per cent. (0·842 sp. gr.; see *Journ. Roy. Mic. Soc.*, 1883, p. 305); DUVAL, alcohol of 36° (= 90 per cent., *Journ. de Microgr.*, 1888, p. 197). The question may now be considered to be finally settled by experiments, specially directed to the clearing up of this point, made by BUSSE (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 49). Busse finds that *alcohol of about 85 per cent.* is the best, *both as regards the cutting consistency and the transparency* of the mass. Care must be taken to keep the mass moist whilst cutting, as it dries by evaporation very quickly.

Lastly, the mass may be *frozen*. After preliminary hardening by alcohol it is soaked for a few hours in water in order to get rid of the greater part of the alcohol (the alcohol should not be removed entirely, or the mass may freeze too hard). It is then dipped for a few moments into gum mucilage in order to make it adhere to the freezing plate, and is frozen. The sections are brought into warm water. If the mass have frozen too hard, cut with a knife warmed with warm water.

A paper has been lately written by FLORMAN (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 184) to recommend that the definitive hardening should be done without the aid of alcohol or chloroform, by simply cutting out the blocks, turning them over, and carefully continuing the evaporation process in the way described above. No doubt the author is right in claiming for this process a superior degree of hardening of the mass; but I doubt whether it is possible to carry the hardening much beyond the point attained by the chloroform or alcohol method without incurring a degree of shrinkage that must be destructive of the scientific value of the preparations.

302. Preservation.—The hardened blocks of collodion may be preserved till wanted in weak alcohol (70 per cent.). They may also be preserved dry by dipping them into melted paraffin (APÁTHY, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 45).

Reference numbers may be written with a soft lead pencil on the bottom of the paper trays, or with a yellow oil pencil on the bottom of the watch-glasses in which the objects are imbedded. On removal of the paper from the collodion after hardening the numbers will be found impressed on the collodion.

303. Cutting.—If the object has not been stained before imbedding, it will form so transparent a mass with the collodion that the arrangement of the object and sections in the right position may be rendered very difficult. It is, therefore, well to stain the collodion lightly, just enough to make its outlines visible in the sections. This may be done by adding picric acid or other suitable colouring matter dissolved in alcohol to the collodion used for imbedding, or to the bergamot oil used for clearing.

To fix a collodion block to the microtome, proceed as follows. Take a piece of soft wood, or, for very small objects, pith, of a size and shape adapted to fit the holder of the microtome. Cover it with a layer of collodion, which you allow to dry. Take the block of collodion, cut a slice off the bottom, so as to get a clean surface; wet this surface first with absolute alcohol, then with ether (or allow it to dry), place one drop of *very thick* collodion on the prepared wood or pith, and press down *tightly* on to it the wetted or dried surface of the block of collodion. Then throw the whole into weak (70 per cent.) alcohol for a few hours (or even less), or into chloroform for a few minutes, in order that the joint may harden.

Dr. Lindsay Johnson informs me that he finds it very convenient to take for this purpose the cement used by metal turners for fastening metal objects on to boxwood chucks. The exact composition of this cement varies somewhat, but an average one is—beeswax, 1 part; rosin, 2 parts. To use it, you must get the block of celloidin perfectly dry at the bottom, then warm the object-holder slightly, if possible, over a flame; drop on to it a few drops of melted cement, and press on to it the block of celloidin, which will be firmly fixed as soon as the cement is cool—that is, in a few seconds.

For objects of any considerable size it is important not to use cork for mounting on the microtome, especially if the object-holder be a vice; for cork bends under the pressure of the holder, and the elastic collodion bends with it, deforming the object. I have seen large embryos so deformed in this way that the sections obtained were true calottes, segments of a sphere. If the object-holder be of the cylinder type, as in the later forms of the Thoma microtome, the above-described

accidents will be less likely to happen, and a good cork may be used; but even then, I think, wood is safer.

Sections are cut with a knife kept abundantly wetted with alcohol (of 50 to 85 or even 95 per cent.). Some kind of drip arrangement will be found very useful here. Apáthy recommends that the knife be smeared with yellow vaselin; it cuts better, is protected from the alcohol, and the mobility of the alcohol on the blade is lessened.

The knife is set in as oblique a position as possible.

Very brittle sections may be collodionised as explained above (§ 282).

The sections are either brought into alcohol (of 50 to 85 or 95 per cent.) as fast as they are made; or if it be desired to mount them in series they are treated according to one of the methods described below, in the chapter on "Serial Section Methods."

The above process is the one that has been until lately the usual one; but some workers prefer not to cut in the wet way with alcohol, as above described, but to clear before cutting. Bumpus (*Amer. Natur.*, xxvi, 1892, p. 80; see *Journ. Roy. Mic. Soc.*, 1892, p. 438) advises clearing the mass, after hardening in chloroform, with white oil of thyme or other suitable clearing agent (see below, § 305). After clearing, the under surface of the block of mass is washed with ether, and cemented with thick celloidin solution to a block of wood for cutting in the manner described above, the whole being thrown into chloroform for a few minutes to harden the joint. The knife is wetted with the clearing oil, and the same oil is employed for covering the exposed surface of the object after each cut. Similar recommendations are made by Eyclesheimer (op. cit., pp. 354, 563), carbolic acid, or glycerin, or the mixture given § 305 being suggested for clearing. Professor Gilson writes me that he has for some time past adopted the practice of clearing before cutting with cedar oil, as described above, § 301. I have not had the opportunity of trying this modification of the usual process. It certainly wears the look of being, at least for objects that have been stained before imbedding, distinctly an improvement.

304. Staining.—The sections may now be stained as desired, either loose, or mounted in series on slides or on paper as

described below. It is *not* in general necessary, nor indeed desirable, to remove the mass before staining, as it usually either remains colourless or gives up the stain on treatment with alcohol. But some of the anilin dyes and some other colours stain it strongly, and are not removed with sufficient completeness by the processes of dehydration and clearing. If it be desired to employ these, the mass may be removed by treating the sections, either loose in a watch-glass, or mounted on a slide with Mayer's albumen (which will, however, generally hold the stain just as obstinately as the collodion), with absolute alcohol or ether.

305. Clearing and Mounting.—You may mount in glycerin without removing the mass, which remains as clear as glass in that medium.

You may mount in balsam, also without removing the mass, which does no harm, and serves the useful purpose of holding the parts of the sections together during the manipulations. Dehydrate in alcohol of 95 or 96 per cent. (not absolute, as this attacks the collodion). Nikiforow (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 189) recommends a mixture of equal parts of alcohol and chloroform. Clear with a substance that does not dissolve collodion. The clearing agents most recommended are origanum oil (*Ol. Origan. Cretici* should be taken, not *Ol. Orig. Gallici;* but see, as to this reagent, the remarks in Chap. XVIII, § 348), bergamot oil (said to make sections shrink somewhat), oil of sandal-wood, lavender oil, oil of cedar-wood (safe and gives excellent results, but acts rather slowly), chloroform, xylol or benzin (may make sections shrink if not well dehydrated), or Dunham's mixture of three or four parts of white oil of thyme with one part of oil of cloves. (As to oil of thyme, see also "Origanum Oil" in Chap. XVIII, § 348.)

Some specimens of clove oil dissolve collodion very slowly, and may be used, but I would not be understood to recommend it. The action of origanum oil varies much, according to the samples; some sorts do not clear the collodion, others dissolve it, others pucker it. Minot (*Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 175) says that Dunham's mixture "clarifies the sections very readily and softens the celloidin just enough to prevent the puckering, which is so annoying with thyme alone."

Carbolic acid has been recommended. Weigert (*Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 480) finds that a mixture of 3 parts of xylol with 1 part of carbolic acid (anhydrous) clears well. But it must not be used with the

basic anilin stains, as it discolours them. For these, anilin oil may be used with the xylol, in the place of carbolic acid.

Anilin oil clears well (it will clear from 70 per cent. alcohol), but unless thoroughly removed the preparation becomes yellowish brown. It may be removed by soaking in chloroform for twenty-four hours (see VAN GIESON, *Amer. Mon. Mic. Journ.*, 1887, p. 49, or *Journ. Roy. Mic. Soc.*, 1887, p. 519, for a review of these clearing agents; see also § 353).

Beechwood kreasote has been recommended (by M. Flesch).

EYCLESHEIMER (*Amer. Nat.*, xxvi, 1892, p. 354; *Journ. Roy. Mic. Soc.*, 1892, p. 565) advises a mixture of equal parts of bergamot oil, cedar oil, and carbolic acid.

306. Double Imbedding in Collodion and Paraffin.—The best-hardened collodion masses are of a more elastic consistency than is desirable for fine section cutting. This defect may be to a certain extent remedied as follows:—Dip the block of hardened collodion into chloroform, and imbed it simply in paraffin.

KULTSCHIZKY'S **Celloidin-Paraffin Method.**—A more thorough-going procedure is the following *infiltration* method (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 48):—After the collodion bath, the object is soaked in oil of origanum (*Oleum Origani vulg.*). It is then brought into a mixture of origanum oil and paraffin, heated to not more than 40° C., and lastly into a bath of pure paraffin.

The mass may be preserved in the dry state, and may be cut dry.

RYDER (*Queen's Micr. Bull.*, 1887, p. 43; *Journ. Roy. Mic. Soc.*, 1888, p. 512) has modified the process by substituting chloroform for the origanum oil. He states that sections chain more easily than even in ordinary paraffin imbedding. They may be cleared for mounting either with chloroform or with Weigert's xylol and carbolic acid mixture (*supra*, § 305; Ryder says "equal parts," but Weigert's formula is as above given).

IDE (*La Cellule*, vii, 1891, p. 347; see also *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 213) has employed with success the following method:—The object is imbedded in collodion in a tube by GILSON'S process (*supra*, § 301); the collodion is boiled for forty minutes, then brought for fifteen minutes (this is for small objects) into chloroform heated to 30° C. containing one fourth part of paraffin dissolved in it, then for ten minutes into pure melted paraffin.

Other Evaporation Masses.

307. Joliet's Gum and Glycerin Method (*Arch. Zool. exp. et gén.*, x, 1882, p. xliii; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 890).—Pure gum arabic dissolved in water to the consistency of a thick syrup. (Solutions of gum sold under the name of strong white liquid glue [*"colle forte blanche liquide à froid"*] may also be used; they have the advantage of having a uniform consistency.*) Pour a little of the solution into a watch-glass, so as not quite to fill it, add from 6 to 10 drops of pure glycerin, stir until thoroughly mixed.

Between the limits of 6 to 10 drops of glycerin the proportions most suitable to the nature of the object and to the season of the year must be found by experimental trials. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

It is often well to soak the object in glycerin before putting it into the mass. In this case less glycerin should be added to the gum, in proportion to the amount of glycerin contained in the object.

The object is imbedded in the mass in the watch-glass, and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature. The block may be preserved in good condition almost indefinitely, the gum, when mixed with a sufficient quantity of glycerin, never becoming hard or brittle. It is generally better to wait till the blocks have assumed such a consistency that they cannot be easily bent. It is after having waited almost a week that the author always obtained the best sections. The gum is dissolved out from the sections by means of a drop of water on the slide. The sections are then covered, and a drop of glycerin being added, the preparation is complete as soon as the water has evaporated.

This mass has the advantage of being transparent. It can be cut dry. Joliet employed it for *Pyrosoma*. A similar mass was employed by Hertwig for *Ctenophora* (*Jen. Zeitsch.*,

* It is highly probable that these commercial preparations contain gelatin, and perhaps some other gum besides gum arabic.

xiv, 1880, pp. 313, 314; *Journ. Roy. Mic. Soc.* [N.S.], ii, p. 278).

It would probably be advantageous to add some preservative substance.

308. ROBERTSON'S **Grape-sugar Method** (*Journ. of Anat. and Physiol.*, xxiv, 1890, p. 230; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 33).

Grape-sugar	5 parts.
Dextrin	10 ,,
Boric acid	1 part.

Mix and add three drachms of water for each quarter of an ounce of the mixture, and dissolve by heating to boiling-point. After cooling, add 6 drops of carbolic acid for each ounce of the solution. The directions for using this mass are practically identical with those given by Joliet for his gum and glycerin mass, last section.

The mass above described is advised for fresh tissues. For hardened tissues a mass is given containing two parts of soft soap (sapo mollis) instead of the 1 part of boric acid; the rest as before.

309. Stricker's Gum Method (*Hdb. d. Gewebel.*, p. xxiv).—A concentrated solution of gum arabic. The object may be prepared in alcohol and imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut.

I have seen masses of admirable consistency prepared by this simple method.

310. Hyatt's Shellac Method (*Am. M. Micr. Journ.*, i, 1880, p. 8; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 320).—Prepare the object by soaking in alcohol, and then put it for a day or two into a clear alcoholic solution of shellac. Take a cylinder of soft wood, split it, and make a groove in one or both of the half-cylinders sufficiently large to admit the object without pressure. Imbed in the groove with plenty of thick shellac solution, and tie together the two halves of the cylinder with thread. In a day or two the shellac will be quite hard; the cylinder is then fixed in a microtome, is soaked with warm water, and sections made. Should the shellac prove so opaque as to interfere with a proper examination of the sections, a drop of borax solution will immediately remove this difficulty.

This process is intended for the purpose of making sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

311. Von Koch's Copal Method (*Zool. Anz.*, 2, vol. i, 1878, p. 36).—Small pieces of the object are stained in bulk and dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained, and filtering. The objects are brought into a capsule filled with the copal solution. The solution is now

slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile, in order that they may more quickly become hard. When they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards, to a slide, by means either of Canada balsam or copal solution. The slide is put aside for a few days more on the warmed tile. As soon as the cement is perfectly hard the sections are rubbed down on a grindstone, and then on a hone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan, after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it. In this way von Koch was able to demonstrate the most delicate lamellæ of connective tissue in *Isis elongata*.

This method was imagined in order to enable the hard and soft parts of corals to be studied in their natural relations. It is evidently applicable to the study of any structures in which hard and soft parts are intimately combined. It is certainly a method of the very greatest value.

312. Ehrenbaum's Colophonium and Wax Method (*Zeit. f. wiss. Mik.*, 1884, p. 414).—Ehrenbaum recommends that the objects be penetrated by a mass consisting of ten parts of colophonium to one of wax. The addition of wax makes the mass less brittle. Sections are obtained by grinding in the usual way. The mass is removed from them by means of turpentine followed by chloroform.

313. Weil's Canada Balsam Method (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 200).—Balsam heated till brittle when cold, then dissolved in chloroform. Heat the objects in the mass on a water-bath. For further details see *Journ. Roy. Mic. Soc.*, 1888, p. 1042.

White of Egg Masses.

314. The White of Egg Method consists in imbedding in a white of egg emulsion, and hardening by alcohol or by the combined action of alcohol and heat. The method had certainly a *raison d'être* at one time, as giving results which could not then be obtained by other means. But the method was extremely cumbrous, had many defects, and must now be considered to be entirely superseded. See *Zeit. f. wiss. Mik.*, 1884, p. 223 (RUNGE); *Morph. Jahrb.*, Bd. ii, 3tes Heft, 1876, p. 445 (CALBERLA); *Zool. Anz.*, 6, vol. i, 1878, p. 130 (SELENKA); *Journ. Roy. Mic. Soc.*, 1883, p. 304 (THOMA); and BECKER'S *Zur Anat. d. ges. u. kranken Linse* (RUGE).

Congelation Masses.

315. The Freezing Method.—Fresh tissues may be, and are, frequently frozen without being included in any mass, and in certain cases very satisfactory sections can be obtained in this manner. But the formation of ice crystals frequently causes tearing of delicate elements, and it is better to infiltrate the tissues with a mass that does not crystallise in the freezing mixture, but becomes hard and tough. Gum arabic affords such a mass. Some workers use common gum water, which is either poured into the well of the microtome or round the object on the object plate, according to the form of microtome used.

316. Syrup and Gum Congelation Mass (HAMILTON, *Journ. of Anat. and Phys.*, xii, 1878, p. 254).—Hamilton cuts sections (of hardened brain) in a Rutherford's freezing microtome. The hardening reagent having been soaked out by water, the tissues are prepared for freezing in the following manner, which it is important to observe, otherwise it will be found that the crystals of ice so break up the delicate nervous tissue as to render it totally useless for minute examination. The tissues are to be well soaked in syrup. The sugar somewhat retards the freezing, and besides, seems to alter the manner of crystallisation, so that instead of the ice being spicular in form it becomes granular, and does no injury to the parts.

The syrup requires to be of a particular strength, viz. double refined sugar, 2 ounces; water, 1 fluid ounce.

Wash the superfluous syrup from the surface, and put into the ordinary mucilage for an hour or so before cutting. Imbed in the freezing microtome with mucilage in the usual way. Float the sections into water.

317. Gum and Syrup Congelation Mass (Cole, *Methods of Microscopical Research*, 1884, p. xxxix; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 318).—Gum mucilage (B. P.), 5 parts; syrup, 3 parts. (For brain and spinal cord, retinæ, and all tissues liable to come in pieces put 4 parts of syrup to 5 of gum.) Add 5 grains of pure carbolic acid to each ounce of the medium.

(Gum mucilage [B. P.] is made by dissolving 4 ounces of picked gum acacia in 6 ounces of water.)

The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of water and boiling.

This medium is employed for soaking tissues previous to freezing. They may remain in it for "any length of time, all the year round" if desired.

The freezing is conducted as follows:—the gum and syrup is removed from the *outside* of the object by means of a cloth; the spray is set going and a little gum mucilage painted on the freezing plate; the object is placed on this and surrounded with gum mucilage; it is thus saturated with gum and syrup, but surrounded when being frozen with mucilage only. This combination prevents the sections from curling up on the one hand, or splintering from being too hard frozen on the other. The mass ought to cut like cheese. Should freezing have been carried too far, wait for a few seconds.

318. Dextrin Congelation Mass (Webb, *The Microscope*, ix, 1890, p. 344; *Journ. Roy. Mic. Soc.*, 1890, p. 113).—Thick solution of dextrin in solution of carbolic acid in water (1 in 40). Use heat for making the solution if desired. This medium is much cheaper than the gum and syrup mass, and, according to Webb, possesses superior cutting qualities.

319. Gelatin Congelation Mass (Sollas, *Quart. Journ. Mic. Soc.*, xxiv, 1884, pp. 163, 164; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 316).—"Instead of gum one uses gelatin jelly. This is prepared and clarified in the usual manner. It should set into a stiff mass when cold. . . . . The tissue to be cut is transferred from water to the melted jelly, and should remain in it till well permeated."

The sections are transferred to a slide as soon as cut. On touching the glass they adhere to it. When enough sections have been thus arranged they are covered with a drop of

glycerin; a cover is put on, and the mount closed with any suitable cement. In process of time the glycerin will permeate the gelatin and convert it into glycerin jelly; this may be hastened by placing the slide in an oven kept at about 20° to 30° C.

320. Gum-Gelatin Congelation Mass (JACOBS, *Amer. Natural.*, 1885, p. 734; *Journ. Roy. Mic. Soc.*, 1885, p. 900).—Gum arabic, 5 parts; gum tragacanth, 1 part; gelatin, 1 part. Dissolve in enough warm water (containing one sixth of glycerin) to give a mass of the consistency of thin jelly when cold.

321. White of Egg Congelation Mass (ROLLETT, *Denkschr. math. naturw. Kl. k. Acad. Wiss. Wien*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 92).—Small portions of tissue brought in the white of a freshly laid egg on to the freezing stage, frozen, and cut. The knife must be well cooled.

322. Oil of Aniseed Congelation Mass (KÜHNE, *Centralb. f. Bakteriol.*, xii, 1892, p. 28; *Journ. Roy. Mic. Soc.*, 1892, p. 706).—Soak in oil of aniseed for twelve to twenty-four hours, freeze and cut, and remove the oil from the sections by means of alcohol.

CHAPTER XVII.

SERIAL SECTION MOUNTING.

323. Choice of a Method.—All the following methods are excellent if properly carried out. I recommend for general work the following:—For paraffin sections that have been already stained, Schällibaum's collodion. For paraffin sections that are to be stained on the slide, Mayer's albumen, unless the stain to be employed be one that will stain the albumen or if the sections be badly folded, in which case take one of the water or alcohol methods given in the next section. For collodion objects, one of the forms of Summers' ether-vapour process. For very large collodion sections, Weigert's process.

Methods for Paraffin Sections.

324. The Water or Alcohol Method.—The principle of this method is due to Gaule (*Arch. f. Anat. u. Phys.* [*Phys. Abth.*], 1881, p. 156), who practised it as follows:—A slide is moistened with alcohol, the sections are arranged on it by means of a camel-hair brush, also moistened with alcohol; the slide is slightly warmed so as to cause the sections to stick to the slide; a cover is put on, and a solution of Canada balsam in xylol (equal parts of each) run underneath it. If the sections are not thicker than $\frac{1}{70}$ mm. they will be clear at once, and nothing remains but to refill the cell day by day as the xylol evaporates, in order to have a perfect mount. If, however, the sections are thicker than $\frac{1}{70}$ mm. they will contain more paraffin than the xylol balsam can dissolve. In that case the excess of paraffin must be removed by means of a drop of pure xylol (the sections being first melted on to the slide as before), and the mount is completed by means of xylol balsam.

Both the moistening with alcohol and the heating are necessary for the attachment of the sections to the slide; the

effect is not obtainable by means of one of these manœuvres alone.

This simple process is very useful for the preliminary examination of trial sections whilst cutting. It is often sufficient to put on a cover, warm, withdraw the melted paraffin by means of a cigarette paper, and run in a drop of clearing agent.

In the primitive form given above, Gaule's method is useful enough for the purpose of mounting a small series of sections that do not require to be stained or otherwise further manipulated on the slide. Later workers have by improvements in the details of the process brought it to a state of great perfection, so that it may now be considered a fairly safe process for extensive series of sections, and will allow of staining on the slide.

Suchannek (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 464) pointed out that the slides must be *absolutely free from grease* in order that the alcohol (50 per cent.) or distilled water, which may be used instead, may spread out in a thin and uniform layer. Secondly, that the slides *should not be warmed to more than* 40° C., it being important both that *the alcohol should evaporate slowly* and that *the paraffin should only be softened, not melted,* until the evaporation is complete. Hoyer had already for this reason advised slow evaporation at the temperature of the laboratory.

Gulland (*Journ. of Anat. and Physiol.*, xxvi, 1891, p. 56; *Journ. Roy. Mic. Soc.*, 1892, p. 161) floats sections on to the surface of warm water (not warm enough to melt the paraffin), or alcohol if preferred, in a dish, and thence floats them into position on the slide. The slide is drained, and the water evaporated from it at a low temperature as described above (Gulland says "a little under 50° C.," which of course is only a low temperature relatively to a paraffin of what I consider an undesirably high melting-point). When the water of the sections has evaporated completely they become more transparent, and look dry. The fixation is then complete, the paraffin may be melted and removed by means of any desired solvent, and the sections may be mounted, or be stained in any medium, or otherwise manipulated as desired. Thin sections will generally be fixed in about an hour; thick ones will require six hours or more. Here it may be pointed out that

the degree of adhesion seems to depend very much on the nature of the sections.

SCHIEFFERDECKER (*Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202) finds that the larger and thinner sections are, the better do they stick, and *vice versâ*. And Mr. ANDREW PRINGLE writes me that he finds that tissues thoroughly fixed in chrome solutions, so that their albuminoid substances have become quite insoluble, do not adhere sufficiently without the aid of some substance to fix them to the slide. He prefers arranging sections on the slide with cold water, and then warming until the sections flatten out.

HEIDENHAIN ("Feschr. Herrn. v. Kölliker gewidm.," &c., 1892; see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 201) also proceeds in this way, and advises that the heating be not carried to above 35° C., at which temperature several hours at least are necessary to ensure fixation. He also finds that water is preferable to alcohol, which is too mobile on the slide and evaporates too quickly. DURHAM'S method (*Quart. Journ. Mic. Sci.*, xxxiii, 1891, p. 116; *Journ. Roy. Mic. Soc.*, 1892, p. 293) is the same as the last described, with the exception that he uses 70 per cent. alcohol instead of water.

325. Schällibaum's Collodion Method (*Arch. f. mik. Anat.*, 1883, p. 565).—One part of collodion is shaken up with three to four volumes (according to the consistency of the collodion) of clove oil or lavender oil. This should give a clear solution. A little is spread thinly on a slide with a small brush. After arranging the sections on the prepared surface, warm over a water-bath, gently, until the clove oil has evaporated (five to ten minutes). The sections are then found to be fixed, and can be treated for days with turpentine, chloroform, alcohol, and watery fluids, without becoming detached. The advantage of this method is that it allows of staining on the slide. If after staining any cloudiness should appear between the sections, dehydrate the slide and treat it several times with absolute alcohol and turpentine, warming it gently the while; or brush the space between the sections repeatedly with a brush moistened with clove oil. This cloudiness only arises from the collodion solution having been taken too concentrated, or having been laid too thick on the slide.

I find it is not necessary to evaporate over a water-bath.

It is sufficient to hold the slide over a spirit lamp until the paraffin has melted and the clove oil has collected in drops between the sections. Schällibaum has stated elsewhere that long evaporation of the slide is necessary if the sections are to be secured firmly enough to allow of staining on the slide. That is not so. What is necessary is that the paraffin and clove oil be thoroughly removed from contact with the sections; and that can be done in a second (as was shown me by Professor v. Korotneff). Warm the slide over a flame, and whilst the paraffin is still melted hold it close before your lips and blow down on it vigorously. The paraffin and clove oil are scattered right and left over the slide, leaving the sections high and dry.

Personally I do not consider Schällibaum's method so safe as Mayer's albumen (and some other methods) for objects that are to be stained on the slide. I recommend it for already stained objects, because it is found to work very pleasantly. Its great defect is that it does not readily lend itself to any device for the flattening out of folded sections. I recommend xylol or naphtha for clearing, in preference to turpentine.

Good collodion is essential in this process.

Strasser (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 45) recommends a mixture of 2 parts collodion, 2 parts ether, and 3 parts castor oil; or (*ibid.*, vi, 2, 1889, p. 153) 2 parts of collodion with one of castor oil, the sections being painted over with a thicker solution, viz. collodium concentratum duplex 2 to 3 parts, castor oil 2 parts, and the slide being plunged at once, without warming, into a bath of turpentine, in which it remains till the paraffin is dissolved (two to ten hours, somewhat less if the whole be put in a stove). The turpentine suffices to harden the collodion (benzin, benzol, and chloroform have the same effect).

Gallemaerts (*Bull. Soc. Belge de Micro.*, xv, 1889, p. 56; *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 493), following Drash, employs a saturated solution of gun-cotton in acetone, diluted to the requisite thinness with absolute alcohol.

Gage prefers preparing slides with a layer of pure collodion, which is allowed to dry, and is rendered adhesive at the instant of using by brushing with clove oil.

Summers (*Amer. Mon. Mic. Journ.*, 1887, p. 73; *Zeit. f. wiss.*

Mik., iv, 4, 1887, p. 482) also employs a dry layer of collodion, which he renders adhesive after the sections are arranged on it, by wetting with a mixture of equal parts of alcohol and ether. As soon as the mixture has evaporated, the sections are found to be fixed.

326. Strasser's Collodion-Paper Method (*Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 346).—This is an extremely complicated modification of Weigert's collodion method for celloidin sections (*post*, § 340). It is certainly too elaborate to have any chance of supplanting, for ordinary work, the classical methods of preparing series on slides. And it is only adapted for use with Strasser's very complicated "Schnitt-Aufklebe-Mikrotom," or automatic section-fixing microtome. The descriptions of the process and the microtome already published by Strasser would fill a small volume, and cannot be properly abstracted in the space at my disposal here. See the original papers in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 346; vi, 2, 1889, p. 154; vii, 3, 1890, p. 290; ib., p. 304; and ix, 1, 1892, p. 8; see also a very short abstract of the last paper in *Journ. Roy. Mic. Soc.*, 1892, p. 703, in which is a figure of the "Schnitt-Aufklebe-Mikrotom."

327. The Shellac Method (Giesbrecht, *Zool. Anz.*, 1881, p. 484).—Prepare a stock of slides covered with a thin and even film of shellac. This is done as follows:—Make a not too strong solution of brown shellac in absolute alcohol, filter it thoroughly; warm the slides, and spread over them a layer of shellac by means of a glass rod dipped in the solution and drawn once over each slide. Let the slides dry.

Just before beginning to cut your sections take a prepared slide and brush it over *very thinly* with kreasote applied by means of a brush; this forms a sticky surface on which the sections are now arranged one by one as cut, care being taken to bring them on to the slide with as little surrounding paraffin as possible.

When all the sections are arranged the slide is heated on a water-bath for about a quarter of an hour at the melting-point of the paraffin; this causes the paraffin to run down into a thin layer, and allows the sections to fall through it and come into close contact with the shellac film, whilst at the same time it evaporates the kreasote.

The slide is allowed to cool, and the sections are now found to be firmly fixed in the shellac. The paraffin is dissolved away by dropping turpentine on to the sections, which are then mounted in Canada balsam. There is no danger of the sections being floated away by the turpentine, because turpentine does not dissolve shellac.

In the note in the *Zool. Anz.* above quoted, the shellac solution is stated to be prepared with common brown shellac (choosing, of course, by preference the paler sorts), on account of the insolubility of white shellac in alcohol. In the *Mitth. d. Zool. Stat.* of Naples, of the same year, "bleached white shellac" is recommended to be dissolved as before, in absolute alcohol. In the *Journ. Roy. Mic. Soc.* (N.S.), vol. ii, 1882, p. 888, it is stated (on hose authority is not clear) that the solution is made by mixing 1 part of

bleached shellac with 10 parts absolute alcohol, and filtering. In the same place it is added that "Dr. Mark uses the bleached shellac in the form in which it is prepared for artists as a 'fixative' for charcoal pictures. It is perfectly transparent, and a film of it cannot be detected unless the surface is scratched. He attaches a small label to the corner of the slide, which serves for the number of the slide and the order of the sections, and at the same time marks the shellac side otherwise not distinguishable." (The latter object is better attained by gumming a paper square, or spinning a ring with ink, in the centre of the unprepared surface of the slide. The disc or ring then serves at the same time for centring the group of sections.)

The account given in the *Mitth. d. Zool. Stat.* further varies in one other detail from that given in the *Zool. Anz.* It directs that the shellac slides be brushed before cutting with *oil of cloves*, instead of kreasote, the slide being slightly warmed before brushing.

The white shellac of commerce is sometimes not easily soluble in alcohol. Kingsley (see Whitman's *Methods in Microscopical Anat.*, p. 117) recommends that brown shellac be taken, and bleached by exposure to the sun.

Caldwell (*Quart. Journ. Mic. Soc.* [N.S.], lxxxvii, 1882, p. 336) simplifies the method by merely brushing over the slide (thinly) at the moment of using with a strong solution of shellac in anhydrous kreasote. (To make the solution, warm the kreasote.)

In both the foregoing methods it often happens that the shellac becomes granular or cloudy on the slide. P. Mayer attributes this to the kreasote or clove oil, and proposes to remedy it by employing carbolic acid instead (*Amer. Natural.*, 1882, p. 733; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 77; *Journ. Roy. Mic. Soc.*, 1885, p. 910). Powdered white shellac is heated with crystallised carbolic acid till it dissolves, and the solution filtered warm.

But more recently (*Intern. Monatschr. f. Anat., &c.*, 1887, H. 2; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 77) the same author, on the ground that hot carbolic acid attacks some tissues, recommends another method. Slides are prepared with alcoholic shellac according to Giesbrecht's plan. The sections are arranged on the dry film and gently pressed down on to it, then exposed for half a minute to vapour of ether.

Chloroform softens shellac; therefore chloroform balsam is not a safe mounting medium for sections fixed by these methods.

These methods do not allow of staining on the slide.

I feel bound to say that I am at a loss to understand by what virtue it is that the shellac method continues to survive, as it certainly seems to do, in the face of far more convenient and efficient processes.

328. Mayer's Albumen (*Mitth. Zool. Stat. Neapel*, iv, 1883; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 317; *Internat. Monatschr. f. Anat.*, 1887, Heft 2; *Journ. Roy. Mic. Soc.*, 1888, p. 160).—White of egg, 50 c.c.; glycerin, 50 c.c.; salicylate of soda, 1 grm. Shake them well together, and filter into a clean bottle.

Fol (*Lehrb.*, p. 134) takes whipped white of egg, filters it

through a Bunsen filter, and adds the glycerin and a little camphor or carbolic acid.

I find it convenient to beat up the egg with a little water before adding the glycerin and filtering; the salicylate should be dissolved in the water in the first instance.

According to my experience carbolic acid is perfectly efficient as a preservative, but is not to be recommended because it precipitates a great deal of the albumen.

A thin layer of the mixture is spread on a cold slide with a fine brush, and the sections laid on it and warmed for some minutes on a water-bath. As the paraffin in the sections melts it carries the albumen away from them, and this is one of the advantages of the method. The sections may be treated with turpentine, alcohol, and aqueous or other stains without any danger of their moving.

It is not *necessary* to warm the slide; the paraffin can be removed in the cold by putting the slide into toluol, xylol, or the like. But the slide should be very thoroughly treated with alcohol after removal of the paraffin, in order to get rid of the glycerin, which will cause cloudiness if not perfectly removed.

The function of the glycerin is merely to keep the layer of albumen moist.

This method allows of the staining of sections on the slide with anilin stains, which is seldom practicable with Schällibaum's method, as the collodion stains with most anilin stains, and does not yield up the colour to alcohol, which the albumen generally does.

This method can be combined with the water process for flattening out sections (§ 280), as described by Henneguy (*Journ. de l'Anat. et de la Physiol.*, 1891, p. 398). A drop of water is spread by means of a glass rod on a slide prepared with white-of-egg mixture, the sections are arranged on it, the whole is warmed (*not* to melting-point of the paraffin) until the sections flatten out, the water is then evaporated off at a temperature of about 40° C., and as soon as it has entirely disappeared the paraffin is melted, and the slide further treated as above described.

According to my experience the albumen method is *absolutely safe*, and is the one that should in general be preferred for staining on the slide, more especially for staining with anilins by Flemming's method.

It has been stated (VOSSELER, *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 457) that Mayer's albumen generally "goes bad," and loses its adhesive power after about six months' keeping. The only remedy for this is to make up fresh mixture every few months, as no change of antiseptics has had any effect in improving its keeping qualities.

329. Flögel's Gum Method (*Zool. Anz.*, 1883, p. 565).—Make a solution of one part gum arabic in twenty parts water, filter, and add a little alcohol to prevent the formation of mould. Slides are prepared by pouring the solution over them, and draining. (It is important that the slides be so perfectly clean as to be evenly wetted all over by the gum solution.) Sections may now be cut and laid on the gum surface before it has become dry, and floated into the proper position; this is the best plan for sections of $\frac{1}{100}$ mm. thickness, and for large sections. For thinner and small sections it is best to take slides that have completely dried, arrange the sections dry on the gum film, and then breathe on it until the gum has become sticky.

A very neat method for cases in which it is not required to treat the slide with watery fluids.

WADDINGTON (*Journ. Quek. M. Club*, vi, 1881, p. 199; *Journ. Roy. Mic. Soc.* [N.S.], i, 1881, p. 704) gives the following process for preparing "arabin," a purified gum arabic which has the advantage of not presenting a granular appearance under the microscope as ordinary gum arabic does.

Dissolve clear and white gum arabic in distilled water to the consistency of thin mucilage. Filter. Pour the filtrate into rectified alcohol, and shake well; the arabin separates as a white pasty mass. Place it on filter-paper, and wash with pure alcohol until the washings are free from water. Dry.

The white powder thus obtained should be dissolved in distilled water and filtered twice. It may then be placed on slides, which are drained, dried, and put away till wanted. In this condition it may be preserved indefinitely.

330. Frenzel's Gum Method (*Arch. f. mik. Anat.*, Bd. xxv, 1885, p. 51).—Gum arabic is dissolved in water to the consistency of a thin mucilage, and to this is added aqueous solution of chrome-alum. An excess of the latter does no harm. Finally add a little glycerin and a trace of alcohol (l. c., p. 142). The slide is prepared with this in the usual way, the sections (either cut dry or in the wet way) are gently pressed on to it with a brush and slightly melted on, and heated for at most a quarter of an hour at a temperature of 30° to 45° C., which

suffices to render the gum insoluble. This layer has the advantage of not staining with the majority of staining fluids; fuchsin and safranin are the only ones that stain it to a harmful degree. In the other anilins, and in carmine or hæmatoxylin, it does not stain. Watery stains (it is stated) may be used with it.

331. Born and Wieger's Quince-Mucilage (*Zeit. f. wiss. Mik.*, 1885, p. 346).—To two volumes of the ordinary pharmaceutical quince-mucilage add one volume of glycerin and a trace of carbolic acid. Spread in a thin layer on a carefully cleaned slide, and arrange the sections on the moist surface. Heat for twenty minutes at a temperature of 30° to 40° C. After removal of the paraffin by turpentine the slide is brought for half an hour into *absolute* alcohol. You may then mount, or pass through successive alcohols, and stain. Alkaline staining fluids must be avoided, as they soften the mucilage and cause the sections to become detached.

332. Gravis's **Agar-agar** (*Bull. Soc. Belge de Micr.*, xv, 1889, p. 72; *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 494).—Solution of agar-agar in 1000 parts of water, to be used as in last section. The formula seems to me superfluous.

333. Gray's **Gelatin Process** (*The Microscope*, ix, 1889, p. 325; *Journ. Roy. Mic. Soc.*, 1890, p. 117).—Solution of gelatin in 100 parts of water. Use as gum arabic solution, taking care not to melt the paraffin; let the slide dry spontaneously overnight, and remove the paraffin with a suitable solvent; remove the solvent with alcohol, and then treat for five minutes with 2 per cent. solution of potassium bichromate to render the gelatin insoluble. Stain as desired, or mount.

Methods for Watery Sections.

334. Fol's Gelatin (Fol, *Lehrb.*, p. 132).—Four grammes of gelatin are dissolved in 20 c.c. of glacial acetic acid by heating on a water-bath and agitation. To 5 c.c. of the solution add 70 c.c. of 70 per cent. alcohol and 1 to 2 c.c. of 5 per cent. aqueous solution of chrome-alum. Pour the mixture on to the slide and allow it to dry. In a few hours the gelatin passes into the insoluble state. It retains, however, the property of swelling and becoming somewhat sticky in presence of water. The slide may then be immersed in water containing the sections, these can be slid into their places, and the whole lifted out; the sections will be found to be fixed in their places.

This method is especially useful for sections made under water, large celloidin sections amongst others.

335. Poli (*Malpighia*, ii, 1888, 2, 3; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 361) arranges sections on a layer of melted Kaiser's gelatin (*supra*, § 294), adds glycerin, and covers. See also *supra*, § 333.

336. Frenzel and Threlfall's Gutta-percha (or Caoutchouc) Method (*Zool. Anz.*, 1883, pp. 51, 301, and 423).—This extremely elegant method is not perfectly safe, the gutta-percha film being liable to tear; and is now, I believe, very generally abandoned.

Methods for Celloidin Sections.

337. Summers' Ether Method (*Amer. Mon. Mic. Journ.*, 1887, p. 73; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 482; *Journ. Roy. Mic. Soc.*, 1887, p. 523).—Besides the method given above (§ 325), which is applicable to celloidin sections, but is needlessly complicated, Summers recommends the following simpler method:—Place the sections in 95 per cent. alcohol for a minute or two, arrange on the slide, and then pour over the sections sulphuric ether *vapour*, from a bottle partly full of liquid ether. The celloidin will immediately soften and become perfectly transparent. Place the slide in 80 per cent. alcohol, or even directly in 95 per cent. if desired. The sections will be found to be firmly fixed, and may be stained if desired.

Schiefferdecker (*Zeit. f. wiss. Mik.*, v, 4, 1888, p. 507) recommends that the slide be one that has been previously prepared with a layer of collodion, if it is desired to stain on the slide; but if not, a clean slide is perfectly sufficient. The slide may of course be treated with ether vapour in a preparation glass or similar arrangement.

338. Apáthy's Oil of Bergamot Method (*Mitth. Zool. Stat. Neapel*, 1887, p. 742; *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 46, and v, 3, 1888, p. 360; *Journ. Roy. Mic. Soc.*, 1888, p. 670).—Cut with a knife smeared with vaselin (§ 303) and wetted with 95 per cent. alcohol. Float the sections, as cut, on bergamot oil (must be green, must mix perfectly with 90 per cent. alcohol, and must not smell of turpentine). The sections spread themselves out on the surface of the oil; before they sink, each one is pushed by means of a needle into its place

on a slip of tracing-paper dipped into the oil. (A good size for the paper is about as broad as the slide, and three times as long as the cover.) When the requisite number of sections has been arranged on the paper, you drain the paper, dry the under side of it with blotting-paper, turn it over, and gently press it down with blotting-paper on to a carefully dried slide. Remove the paper by rolling it up from one end. The sections remain adhering to the slide, and may have the remaining bergamot oil removed from them by means of a cigarette paper. If they are already stained, nothing remains but to add balsam and a cover.

In the case of unstained or very small objects, it is well to add a little alcoholic solution of safranin to the bergamot oil. The celloidin of the sections becomes coloured in it in a few seconds, and makes them readily visible. The colour disappears after mounting in a few days.

If the sections are to be stained, the slide after removal of the bergamot oil is exposed for a few minutes to the vapour of a mixture of ether and alcohol, then brought into 90 per cent. alcohol, and after a quarter of an hour therein may be stained in any fluid that contains 70 per cent. alcohol or more.

If it be desired to stain in a watery fluid, care must have been taken when arranging the sections to let the celloidin of each section overlap that of its neighbours at the edges, so that the ether vapour may fuse them all into one continuous plate. This will become detached from the slide in watery fluids, and may then be treated as a single section.

339. Apáthy's Series-on-the-Knife Method (*Zeit. f. wiss. Mik.*, vi, 2, 1888, p. 168).—The following is in some respects more convenient than the oil of bergamot method. The knife is well smeared with yellow vaselin rubbed evenly on with the finger, and is wetted with alcohol of 70 to 90 per cent. As fast as the sections are cut they are drawn with a needle or small brush to a dry part of the blade, and there arranged in rows, the celloidin of each section overlapping or at least touching that of its neighbours. The rows are of the length of the cover-glass, and are arranged one under the other so as to form a square of the size of the cover-glass. When a series (or several series, if you like) has been thus completed, the sections are dried by laying blotting-paper on them (there is no risk of their becoming attached to it, as they are

held down by the vaselin). The series is then painted over with some of the thickest celloidin solution used for imbedding, is allowed to evaporate for five minutes in the air, and is then either wetted with 70 per cent. alcohol, and allowed to remain whilst cutting is proceeded with, or (if no more sections are to be cut, or if the knife is now full) the knife is removed and brought for half an hour into 70 per cent. alcohol. This hardens the celloidin around the sections into a continuous lamella, which can be easily detached by means of a scalpel, and stained, or further treated as desired. It is well to bring it at once on to a slide, moisten the edges of the celloidin plate with ether and alcohol mixture, so that it may not become detached, and bring the whole into the staining solution.

340. Weigert's Collodion Method (*Zeit. f. wiss. Mik.*, 1885, p. 490).—Sections are cut wet with alcohol. Care should be taken not to have so much alcohol on the knife as to cause the sections to float. Prepare a slip of porous but tough paper (Weigert recommends "closet paper"), of about twice the width of the sections. Soak it in alcohol, take it by both ends, stretch it slightly, and lower it on to the section that is on the knife. The section will adhere to the paper, and is taken up by moving the slip horizontally or slightly upwards, away from the edge of the knife. Take up the first section towards the end of the paper that you hold in your left hand, and let the remaining sections follow in order from left to right. After each section has been taken up, the slip is placed, whilst the next section is being cut, with the sections upwards on a moist surface prepared by arranging several layers of blotting-paper, covered with one layer of closet paper, in a plate, and saturating the whole with alcohol. When all the sections have been arranged on the slip, you pass to the next stage of the process, the collodionisation of the series.

This is done in two steps. The first of these consists in transporting the series on to a plate of glass prepared with collodion. The plate is prepared beforehand by pouring on to it collodion and causing it to spread out into a thin layer, as photographers do, and allowing it to dry. (A number of the plates may be prepared and kept indefinitely in stock;

microscope slides will do for series of small sections.) Take one of these plates; lay the slip of paper with the sections on the plate, the sections downwards; press it down gently and evenly, and the sections will adhere to the collodion, then carefully remove the paper. (Do not place more than one or at most two lines of sections on the same plate, for those first placed run the risk of becoming dry whilst you are placing the others.) This finishes the first stage of the collodionising process.

Now remove with blotting-paper any excess of alcohol that may remain on or around the sections, pour collodion over them, and get it to spread in an even layer. As soon as this layer is dry at the surface you may write any necessary indications on it with a small brush charged with methylen blue (the colour will remain fast throughout all subsequent manipulations).

The plate may now either be put away till wanted in 80 per cent. alcohol, or may be brought into a staining fluid. Weigert recommends his hæmatoxylin process, but other watery stains may be used. The watery fluid causes the double sheet of collodion to become detached from the glass, holding the sections fast between its folds. It is then easy to stain, wash, dehydrate, and mount in the usual way, merely taking care not to use alcohol of more than 90 to 96 per cent. for dehydration. Weigert recommends for clearing the above-described mixture of xylol and carbolic acid (§ 305). Both the dehydration and the clearing take rather longer with the collodionised series than with free sections.

The series should be cut into the desired lengths for mounting whilst in the alcohol. It is perhaps safer to lay them out for cutting on a strip of closet paper saturated with alcohol.

It is hardly necessary to comment on the great value of this beautiful method.

It is suggested by STRASSER that gummed paper might be an improvement on the glass plates used in this process—especially for very large sections. See *ante*, § 326.

Other Methods.

341. Giacomini's Collodion-Gelatin Method.—See the chapter on "Nerve-centres" in Part II.

CHAPTER XVIII.

CLEARING AGENTS.

342. Introductory Remarks.—Clearing agents are liquids one of whose functions it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction not greatly inferior to that of the tissues to be cleared. Hence all clearing agents are liquids of high index of refraction. The same substances have also a second function, which consists in getting rid of the alcohol in which preparations are generally preserved, and facilitating the penetration of the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence all of the group of bodies here called "clearing agents" must be capable of expelling alcohol from tissues, and must be at the same time solvents of Canada balsam and the other resinous mounting media.

It is important to note again, notwithstanding some repetition, the manner of employing these agents. The old plan was to take the object out of the alcohol and float it on the surface of the clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the clearing agent can get into it; hence the object must shrink. To avoid or lessen this cause of shrinkage, clearing is now generally done by the method suggested by Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object. This is done in the following manner. Take a test-tube, and put into it enough alcohol to contain the objects (a watch-glass will often do well, but a test-tube is safer). With a pipette carefully put under the alcohol a sufficient quantity of clearing medium (or carefully pour the alcohol on to the clearing medium). Then put the objects into

the alcohol. They will sink down to the level of separation of the two liquids at once; and after some time they will be found to have sunk to the bottom of the clearing medium. They may then be removed by means of a pipette, or the supernatant alcohol drawn off and the preparations allowed to remain until wanted.

The chief clearing agents are essential oils. A classification of these is given below (No. 343, Stieda and Schiefferdecker).

The penetration of all clearing media may be hastened by using them warm. Directions for clearing are given when necessary under the heads of the different organs and tissues. It will suffice here to advise the beginner to keep on his table the following:—Oil of cedar, for general use; clove oil, for making minute dissections in cases in which it is desirable to take advantage of the property of that essence of forming very convex drops on the slide, and of imparting a remarkable brittleness to soft tissues; carbolic acid, for rapidly clearing imperfectly dehydrated objects.

It frequently happens that the essential oil with which objects are being treated in a watch-glass or on a slide becomes cloudy after a short time, and fails to clear the tissues. This is owing to a combination between the essential oil and moisture, derived, I think, rather from the air than from the objects themselves. The cloudiness can usually be removed by warming (as pointed out by Hatchett Jackson, *Zool. Anzeig.*, 1889, p. 630), but this remedy is not always successful, for in certain states of the atmosphere the cloudiness will persist, notwithstanding continued warming. It is for this reason that I advise that clearing be done, whenever possible, in shallow well-corked tubes, under which conditions the phenomenon rarely occurs.

343. Classification of Clearing Agents (Stieda).—Stieda's experiments with essential oils led him to establish the following classification:

A. The *turpentine* group, capable of clearing in a short time perfectly dehydrated sections, but clearing watery sections only after many hours or not at all.

Ol. Terebinthinæ.	Ol. Balsam. Copaivæ.
Ol. Absinthii.	Ol. Cortic. Aurantiorum.

Ol. Cubebarum.
Ol. Fœniculi.
Ol. Millefolii florum.
Ol. Sassafras.
Ol. Juniperi.
Ol. Menthæ crispæ.
Ol. Origani vulgaris.
Ol. Lavandulæ.
Ol. Cumini.
Ol. Cajeputi.
Ol. Cascarillæ cortic.
Ol. Sabinæ.
Ol. Citri.

This, then, for Stieda, is the *Index Expurgatorius* of clearing media.

B. The *oil-of-cloves* group, clearing *very rapidly* sections that have been dehydrated, and clearing watery sections "somewhat more slowly" and with a certain amount of shrinkage.

Ol. Gaultheriæ.
Ol. Cassiæ.
Ol. Cinnamomi.
Ol. Anisi stellati.
Ol. Bergamotti.
Ol. Cardamomi.
Ol. Coriandri.
Ol. Carui.
Ol. Roris marini.

But Stieda found kreasote preferable to any of these.

He relates that kreasote was suggested to him by a paper of KUTSCHIN, *Über den Bau des Rückenmarks des Neunanges*, Kasan, 1863. Kutschin rinsed his sections *in water*, brought them on to slides, drew off the water by means of blotting-paper, and added a drop of kreasote at the side. When clear, he covered and closed the mounts with a border of dammar.

Stieda modified this process by mounting in dammar instead of kreasote.

He then tried experiments to ascertain whether oil of cloves could be applied in the same manner—that is, to the clearing of non-dehydrated sections. He found that it could, though its employment requires longer time. Sections brought from water into kreasote clear in a few minutes, whilst in oil of cloves they require from half an hour to an hour or more; and this slowness of the process exposes them to the risk of shrinkage.

To the group of good clearing agents should be added—cedar-wood oil, sandal-wood oil, carbolic acid.

NEELSEN and SCHIEFFERDECKER (*Arch. f. Anat. u. Phys.*, 1882, p. 206) examined a large series of ethereal oils (prepared by Schimmel and Co., Leipzig), with the object of finding a

not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, not evaporating too quickly, and not having a too disagreeable smell.

The following is a list of twenty-four products examined by them. It seems worth while to give it, although the authors only found three amongst the number that fulfil the conditions; as to know that they have been found wanting in some of these respects may perhaps save somebody a wild-goose chase.

Oils of Anise, Amber, Birch tar, Cajeput, Calmus, Cassia, Cedar-wood, Citrons, Dill, Field thyme, Fir needles, Mint, Cumin, Niobe, Origanum, Palmarosa, Peppermint, Penny-royal, Rosemary, Sassafras, Spikenard, Thuja, Sandal-wood, Caraway.

Of these, the following three fulfil the conditions and can be *recommended:—Cedar-wood, Origanum, Sandal-wood.*

It would be important to possess a list of the exact indices of refraction of the substances used for clearing. I have, unfortunately, not been able to obtain sufficient information of a trustworthy nature for the compilation of such a list. Cedar oil has nearly the index of crown glass (this is true of the oil in the thick state to which it is brought by exposure to the air, not of the new, thin oil, which is less highly refractive), it therefore clears to the same extent as Canada balsam. Clove oil has a much higher index, and therefore clears more than balsam. Turpentine, bergamot oil, and kreasote have much lower indices, and therefore clear less.

344. Cedar Oil (Neelsen and Schiefferdecker, l. c., last section).—For finest cedar-wood oil, price per kilo varies from fifteen to twenty shillings, say about sevenpence halfpenny per ounce for small quantities, or about the price of clove oil. Very thin, colour light yellow, odour slight (of cedar-wood), evaporates slowly, is not changed by light, is miscible with chloroform balsam, and with castor oil. Clears readily tissues in 95 per cent. alcohol, without shrinkage, does not extract anilin colours. Celloidin sections are cleared in five to six hours.

Cheap, but requires an inconvenient length of time for the clearing of celloidin sections.

The observer should be careful as to the quality of the cedar oil he obtains. I have examined the clearing properties of a sample obtained from the celebrated firm of Rousseau,

Paris. This sample was absolutely colourless. It *totally* failed to clear absolute alcohol objects after many days.

The authors think that a laboratory supplied with cedar oil and origanum oil is fully equipped for all possible cases (the origanum oil being used merely to take the place of cedar-wood oil for the special case of celloidin sections). See below, § 348.

Cedar oil is very penetrating, and for this and other reasons is one of the best media, if not the very best, for preparing objects for paraffin imbedding.

345. Clove Oil.—Samples of clove oil of very different shades of colour are met with in commerce. It is frequently recommended that only the paler sorts should be employed in histology. A word of explanation is here necessary. Doubtless it is, in general, best to use a pale oil, provided it be pure, but it is not always easy to obtain a light-coloured oil that is pure. Clove oil passes very readily from yellow to brown with age, so that in choosing a colourless sample you run great risk of obtaining an adulterated sample, for clove oil is one of the most adulterated substances in commerce.

Two important properties of clove oil should be noticed here. It does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops. This property makes it a very convenient medium for making minute dissections in. The second property I wish to call attention to is that of making tissues that have lain in it for some time very brittle. This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

Clove oil has, I fancy, the highest index of refraction of all the usual clearing agents; it clears objects *more* than balsam. It dissolves celloidin (or collodion), and therefore should not be used for clearing sections cut in that medium, without special precautions. Notwithstanding the opinion of Schieffer-decker, I consider this to be one of the best of clearing agents, and very valuable on account of the properties to which attention has been called above. New clove oil washes out anilin colours more quickly than old. It is well to possess trustworthy samples of both new and old oil.

346. Cannel Oil.—Greatly resembles clove oil, but is in general thinner. An excellent medium, which I particularly recommend.

347. Oil of Bergamot.—SCHIEFFERDECKER (*Arch. Anat. u. Phys.*, 1882 [Anat. Abth.], p. 206) finds that this oil has many good qualities; it clears 95 per cent. alcohol preparations and celloidin preparations quickly, does not attack anilin colours, but the strong odour is disagreeable; it is as dear as oil of cloves, twice as dear as oil of origanum, and three times as dear as oil of cedar. He considers its action preferable to that of oil of cloves, but, all things considered, gives the palm to cedar and origanum. I think that this is a very valuable medium, and though I do not agree with Schiefferdecker in thinking its action superior to oil of cloves, I think it should always be kept at hand.

Bergamot oil is, I believe, the least refractive of these essences, having a lower index than even oil of turpentine.

SUCHANNEK (*Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 158) says that bleached, colourless bergamot oil will not take up much water, whereas a green oil will take up as much as 10 per cent.

348. Oil of Origanum (NEELSEN and SCHIEFFERDECKER, *Arch. Anat. u. Phys.*, 1882, p. 204).—Price per kilo 15 marks (=15*s.*). Thin, light brown colour, odour not too strong, agreeable, does not evaporate too quickly, is not changed by light, is miscible with chloroform balsam and with castor oil. Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

For work with celloidin sections care should be taken to obtain *Ol. Origani Cretici* ("Spanisches Hopfenöl"), not *Ol. Orig. Gallici* (v. GIESON; see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 482). Specimens of origanum oil vary greatly in their action on celloidin sections, and care should be taken to obtain a good sample.

SQUIRE, in his *Methods and Formulæ*, &c., p. 81, says that origanum oil (meaning, doubtless, the commercial product) is nothing but oil of white thyme more or less adulterated, and that the product sold as Ol. Origani Cretici is probably oil of marjoram.

349. Sandal-wood Oil (NEELSEN and SCHIEFFERDECKER, *ibid.*). —"Finest East Indian sandal-wood oil," price per kilo 50 marks (= £2 10*s.*). Somewhat thicker than the last two, light yellow, odour faint, agreeable, evaporation hardly perceptible, unchangeable by light, miscible with chloroform balsam and with castor oil. Ninety-five per cent. alcohol preparations cleared quickly, celloidin more slowly, anilin colours unaffected.

Very useful; its worst fault is its high price.

350. Turpentine.—Generally used for treating sections that have been cut in paraffin, as it has the property of dissolving out the paraffin and clearing the sections at the same time; but many other reagents (naphtha, for instance) are preferable for this purpose (see *ante*, § 283). If used for alcohol objects it causes considerable shrinkage, and alters the structure of cells more than any other clearing agent known to me, unless used in the thickened state, a method which is much liked for some purposes in Germany. Thickened turpentine ("Verhartzes Terpentinöl" of German writers) is prepared by exposing rectified turpentine in thin layers for some days to the air. All that is necessary is to pour some turpentine into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency. Turpentine has, I believe, the lowest index of refraction of all the usual clearing agents except bergamot oil; it clears objects *less* than balsam.

351. Carbolic Acid.—Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. This is a very good medium, but it is better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium. It is, however, a good medium for celloidin sections (see above, § 305).

352. GAGE'S **Mixture** (*Proc. Amer. Soc. Micr.*, 1890, p. 120; *Journ. Roy. Micr. Soc.*, 1891, p. 418).—Carbolic acid crystals melted, 40 c.c.; oil of turpentine, 60 c.c.

352a. Kreasote.—Much the same properties as carbolic acid. *Beech-wood* kreasote is the sort that should be preferred for

many purposes,—for clearing celloidin sections (for which it is a very good medium) amongst others.

353. Anilin Oil.—This is a very important reagent on account of its ability to clear excessively watery objects. Common anilin oil will readily clear sections from 70 per cent. alcohol, and with certain precautions objects may be cleared from watery media without the intervention of alcohol at all. This property renders anilin valuable in certain cases as a penetrating medium for preparing for paraffin imbedding. For ordinary work the usual commercial anilin will suffice; and it is immaterial whether it be colourless or have become brown through oxidation. For difficult work, it is well to use a perfectly anhydrous oil. This may easily be prepared by distilling the common oil (SUCHANNEK, *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 156). It should be distilled over into a perfectly dry bottle containing a few pieces of caustic potash (this, being quite insoluble in anilin, may remain in the anilin without being in any way a hindrance to its employment). The first 10 to 12 c.c. of liquid that come over will contain some water, and should be thrown away. The remainder of the distillate should be preserved over the potash, the bottle being closed with a rubber stopper and kept protected from light as far as possible. It is used for preparing for paraffin in the usual way, alcohol objects clearing quickly in it, whilst quite watery objects will make the oil cloudy and require it to be changed once or twice. SUCHANNEK recommends that the objects be not brought direct from the anilin into paraffin, but first soaked for some hours in toluol or xylol—the anilin in this process taking the place of the usual alcohol rather than that of the usual clearing agent.

As above pointed out (§ 305), anilin is used for clearing celloidin sections, and is sometimes found very valuable for this purpose.

354. Xylol, Benzol, Toluol, Naphtha, Chloroform.—Too volatile to be recommendable as general clearing agents, but may be used for celloidin sections or for paraffin sections. For these I greatly recommend naphtha.

355. Absolute Alcohol (SEILER, *Journ. Roy. Mic. Soc.*, 1882, p. 126).—Absolute alcohol is recommended by Seiler for preparing objects for mount-

ing in balsam, the balsam being in this case dissolved in warm absolute alcohol (see No. 419). The method is said by Seiler to give very good results; but it is obvious that it is only applicable to cases in which it is not desired to make a preliminary examination of the cleared objects (for the sake of selecting the best or the like) before mounting them.

I have several times tried this process, with results in no wise distinguishable from those obtained by ordinary methods, and cannot recommend it for ordinary purposes.

CHAPTER XIX.

INDIFFERENT LIQUIDS—EXAMINATION AND PRESERVATION MEDIA.

356. Introductory.—I comprehend under this heading all the media in which an object may be examined. The old distinction of "indifferent" liquids, and those which have some action on tissues, appears to be misleading more than helpful; inasmuch as it is now well understood that *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism; and this plasma itself is only "indifferent" whilst all is *in situ;* as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become evidently artificial.

It does not appear necessary to create a separate group for mounting media, as all preservative media may be used for mounting.

For directions as to making permanent mounts in fluid media, see the early sections of Chap. XX.

357. Water.—To preserve it from mould, a lump of thymol or camphor should be kept in the supply. Water may be employed without inconvenience, and sometimes (on account of its low index of refraction, with great advantage) for the examination of all structures that have been fixed with osmic or chromic acid, or some salt of the heavy metals; but it is by no means applicable to the examination of fresh tissues—that is, tissues that have not been so fixed. It is important that the beginner should bear in mind that water is very far from being an "indifferent" liquid; many tissue-elements are greatly changed by it (nerve-end structures, for instance), and some are totally destroyed by its action if prolonged (for instance, red blood-corpuscles).

358. Theory of Indifferent Liquids.—In order to render water inoffensive to such tissues as these it must, firstly, have dissolved in it some substance that will give it a density equal to that of the liquids of the tissue, so as to prevent the occurrence

of osmosis, to which process the destructive action of pure water is mainly due. Salt solution is a medium suggested by this necessity. But salt solution by no means fulfils all the conditions implied in the notion of an "indifferent" liquid. In so far as it possesses a density approaching to that of the liquids of the tissues, one cause of osmosis is eliminated; but there remains another, due to the difference of composition of the liquids within the tissues and that without. Cell contents are a mixture of colloids and crystalloids; salt solution contains only a crystalloid, whose high diffusibility causes it to diffuse over into the colloids of the tissues. In order to reduce the consequent osmotic processes to a minimum, it is necessary that the examination medium contain, in addition to a due proportion of salt or other crystalloid, also a due proportion of colloids. By adding, for instance, white of egg to salt solution this end may be attained, and, as a matter of fact, the liquids recommended as indifferent are found invariably to contain both crystalloids and colloids. Thus (as stated by Frey) vitreous humour contains 987 parts of water to about 4·6 of colloid matters and 7·8 of crystalloids (common salt). In 1000 parts of the juice of fruits are contained about 3·8 parts of colloid matter (albumen), 5·8 of salt, and 3·4 of urea. In blood-serum, 8·5 of colloids and 1 of crystalloid substance are found.

359. Salt Solution ("normal salt solution," "physiological salt solution").—0·75 per cent. sodium chloride in water. Carnoy recommends the addition of a trace of osmic acid.

360. Iodised Serum.—Iodised serum was first recommended by Max Schultze (*Virchow's Archiv,* xxx, 1864, p. 263). I take the following instructions concerning it from Ranvier (*Traité,* p. 76).

The only serum that gives really good results is the amniotic liquid of mammals. A gravid uterus of a sheep or cow having been obtained (in large slaughter-houses such can be obtained without difficulty), an incision is made through the wall of the uterus and the fœtal membranes. A jet of serum issues from the incision, and is caught in a flask prepared for the purpose. Flakes of iodine are then added, and the flask is frequently agitated for some days. Two points should be noted. A perfectly fresh amnios must be taken, for the

merest incipience of putrefaction will spoil the preparation. The flask should have a wide bottom, so that the serum may form only a shallow layer in it; otherwise the upper layers will not be sufficiently exposed to the action of the iodine.

Another method is as follows:—Serum is mixed with a large proportion of tincture of iodine; the precipitate of iodine that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use.

Ranvier explains that at the outset serum dissolves very little iodine; but if an excess of iodine be kept constantly present in the solution, it will be found that after two or three weeks iodides are formed, and allow fresh quantities of iodine to dissolve; so that after one or two months a very strongly iodised serum is obtained. It should be dark brown. Such a solution is the most fitting for the purpose of iodising fresh serum in the manner directed above, and for making the different strengths of iodised serum that are required for different purposes. In general, for maceration purposes, a serum of a pale brown colour should be employed.

361. Artificial Iodised Serum (Frey, *Le Microscope*, p. 131).

Distilled water	135 grms.
White of egg	15 „
Sodium chloride	0·20 grm.
Mix, filter, and add—	
Tincture of iodine	3 grms.

There is formed a precipitate, which is removed by filtering through flannel; and a little iodine is added to the filtrate.

362. Kronecker's Artificial Serum (from Vogt et Yung, *Traité d'Anat. comp. prat.*, p. 473: I have been unable to discover the original source).

Common salt	6 grms.
Caustic soda	0·06 grm.
Distilled water	1000 grms.

363. Migula's **Glycerized Blood-serum** (see the paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 172; also *Journ. Roy. Mic. Soc.*, 1890, p. 804).

364. Aqueous Humour, Fruit Juice, Simple White of Egg.—Require no preparation beyond filtering. They may be iodised if desired.

365. Syrup.—An excellent medium for examining many structures in the fresh state. To preserve it from mould, chloral hydrate may conveniently be dissolved in it (1 to 5 per cent.). I have used as much as 7 per cent., and found no disadvantage.

Carbolised Syrup.—Carbolic acid may be employed instead of chloral; 1 per cent. is sufficient.

Either of these syrups may be used as a mounting medium, but they are not to be recommended for that purpose, as there is always risk of the sugar crystallising out.

A good strength for syrup is equal parts of loaf sugar and water. Dissolve by boiling.

366. Saliva.—Saliva has been recommended with the idea of its being innocuous to delicate structures; it is of course a macerating agent (see MACERATING AGENTS, **Artificial Saliva**).

367. Carbolic Acid.—1 per cent. in water. Is a mounting medium.

368. Kreasote.—5 per cent. in water.

369. Thwaites' Kreasote Fluid (see BEALE, *How to Work, &c.*, p. 55).

370. Beale's Naphtha and Kreasote (*ibid.*, p. 56).

371. Quekett's Wood-naphtha Fluid (*ibid.*).

372. Alum Sea-water.—A saturated solution of alum in sea-water is useful for the examination and preservation of the tissues of many marine organisms (Medusæ, Siphonophora, Ctenophora, Pelagic Tunicata). The animals may be killed in the fluid, which is a fair fixing agent.

373. Acetate of Alumina (GANNAL'S SOLUTION, BEALE, *ibid.*).

Acetate of alumina	1 part.
Water	10 parts.

374. Acetate of Potash (MAX SCHULTZE, *Arch. mik. Anat.*, vii, 1872, p. 180).—A nearly saturated solution in water. It is used by letting a drop run in under the cover-glass to the object, which is in water. After twenty-four hours the mount may be closed. The index of refraction is lower than that of glycerin.

This medium has been frequently recommended as having the property of preventing the blackening of objects that have been treated with osmium; but it seems extremely doubtful whether this is really the case.

375. Calcium Chloride (*Micro. Dict.*, art. "*Calcium*, chloride").—Either about 1 part of the salt to 2 of water, or a saturated solution may be used. A lump of camphor should be added to the solution to preserve it.

As this salt is very hygroscopic, its solution presents the advantage of not drying up, so that it is not necessary to close the mounts until it is desired to put them away.

376. Chloral Hydrate.—5 per cent. in water (LAVDOWSKY, *Arch. f. mik. Anat.*, 1876, p. 359).

Or, 2·5 per cent. in water (BRADY, *British Copepods*).

Or, 1 per cent. in water (MUNSON, *Journ. Roy. Mic. Soc.*, 1881, p. 847).

377. Hydrochlorate of Chinolin appears to be a powerful antiseptic (see ROSENTHAL, in *Biol. Centralbl.*, ix, 1890, p. 767; or *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 342).

378. Alcohol.—Not very recommendable for mounting, as if taken weak it is not a very efficient preservative, and if taken strong it attacks the cement of mounts.

CARPENTER (*The Microscope*) recommends a strength of 1 part to 5 of water.

The chief use of alcohol for preservation purposes is of course for preserving specimens in till wanted for further preparation and study. See, on this point, the remarks in Chap. I, § 2.

Mercurial Liquids.

379. Corrosive Sublimate Solution (HARTING'S FLUID, *Micro. Dict.*, art. "Preservation," p. 640).—One part of sublimate to from 200 to 500 of water. (For blood-corpuscles of frog 1—400, of birds 1—300, of mammals 1—200.) "Harting recommends this as the best preservative for the corpuscles of the blood, nerve, muscular fibre, &c."

380. Pacini's Fluids (*Journ. de Mic.*, iv, 1880; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 702).—Pacini remarks that "bichloride of mercury coagulates and precipitates the albuminous matter that exists in the interstitial fluids of the tissues," and therefore in order to prevent this coagulation it is well to associate with it salt for certain preparations, or acetic acid for others. On this principle are prepared the following classical fluids of Goadby and Pacini:

FLUID No. 1 is identical with that of Harting given above, viz. 1·200 sublimate in water. Pacini uses it for removing, when desired, the salt or acid from preparations that have been placed in one of the other solutions.

FLUID No. 2:

Bichloride of mercury	1 part.
Common salt	2 parts.
Water	200 „

Of general employment, but especially useful for blood-corpuscles of cold-blooded animals, as it has a less density than the following fluid. It pre-

serves spermatic fluid, epithelia, nerves, and muscle-fibres. It is also used for fixing Infusoria, a small quantity being added to the water containing them.

FLUID No. 3:

Bichloride of mercury	1 part.
Common salt	4 parts.
Water	200 „

For blood-corpuscles of warm-blooded animals.

FLUID No. 4:

Bichloride of mercury	1 part.
Acetic acid	2 parts.
Water	300 „

"Serves best for the nuclei of animal tissues, but it swells up the fibres and distorts the forms of the cells."

FLUID No. 5 (FREY, *Le Microscope*, 1867, p. 233).—In the place here quoted, Frey speaks of the liquids of Pacini as differing from those of Goadby through their containing glycerin in lieu of alum. He gives the following directions. Take—

Sublimate	1 part.
Sodium chloride	2 parts.
Glycerin (25° Baumé)	13 „
Water	113 „

Allow the mixture to remain undisturbed for at least two months. At the end of that time take for use 1 part, mix with 3 parts of water, and filter. This mixture is said to be a good preservative of all delicate tissues.

FLUID No. 6 (*ibid.*):

Sublimate	1 part.
Acetic acid	2 parts.
Glycerin (25° Baumé)	43 „
Water	115 „

This mixture is to be employed in the same way as the last. It is said to destroy red blood-corpuscles, but to preserve white blood-corpuscles.

381. Modifications of the foregoing Sublimate Solutions.—The following formulæ are quoted by Frey from Cornil as being in use at the Pathological Institute of Berlin:

1.

Sublimate	1 part.
Sodium chloride	2 parts.
Water	100 „

For the more vascular tissues of warm-blooded animals.

2.

Sublimate	1 part.
Sodium chloride	2 parts.
Water	200 „

For similar tissues of cold-blooded animals.

3.

Sublimate	1 part.
Sodium chloride	1 „
Water	300 parts.

For pus-corpuscles and analogous elements.

4.	Sublimate	1 part.
	Water	300 parts.

For blood-corpuscles.

5.	Sublimate	1 part.
	Acetic acid	1 „
	Water	300 parts.

For epithelia, connective tissue, and pus-corpuscles, when it is desired to demonstrate the nuclei.

6.	Sublimate	1 part.
	Acetic acid	3 parts.
	Water	300 „

For ligaments, muscles, and nerves.

7.	Sublimate	1 part.
	Acetic acid	5 parts.
	Water	300 „

For glandular tissues.

8.	Sublimate	1 part.
	Phosphoric acid	1 „
	Water	30 parts (*sic*).

For cartilaginous tissues.

382. Goadby's Fluids (*Micro. Dict.*, art. "Preservation").

1ST FLUID:

Bay salt (coarse sea salt)	4 ounces.
Alum	2 „
Corrosive sublimate	2 grains.
Boiling water	1 quart.

This is found to be "too strong" for most purposes, and therefore the following is recommended for general purposes.

2ND FLUID:

Bay salt	4 ounces.
Alum	2 „
Corrosive sublimate	4 grains.
Water	2 quarts.

"Schultze recommends it for preserving *Medusæ, Echinodermata,* Annelid larvæ, *Entomostraca, Polythalamia,* and *Polycystina,* and advises the use of glycerin afterwards to produce transparence."

3RD FLUID.—When carbonate of lime exists in the preparations, the alum must be omitted. The following formula is recommended:

Bay salt	8 ounces.
Corrosive sublimate	2 grains.
Water	1 quart.

4TH FLUID.—"Marine animals require a stronger fluid of this kind, made by adding about 2 ounces more salt to the last."

383. Owen's Fluid (quoted from VOGT et YUNG, *Traité d'Anat. comp. pratique*, p. 19).

Corrosive sublimate . . .	0·014 grm.
Alum	79 grms.
Salt	137 „
Water	1680 „

Said to be very useful for the preservation of soft-bodied animals.

384. Gilson's Fluid (CARNOY'S *Biologie cellulaire*, p. 94).

Alcohol of 60 per cent. . . .	60 c.c.
Water	30 „
Glycerin	30 „
Acetic acid (15 parts of the glacial to 85 of water)	2 „
Bichloride	0·15 grm.

A really excellent medium for the study of fine cellular detail with well-fixed objects.

385. Gage's Albumen Fluid (*Zeit. f. wiss. Mik.*, 1886, p. 223).

White of egg	15 c.c.
Water	200 „
Corrosive sublimate . . .	0·5 grm.
Salt	4 grms.

Mix, agitate, filter, and preserve in a cool place. Recommended for the study of red blood-corpuscles and ciliated cells.

Other Fluids.

386. Chloride and Acetate of Copper (RIPART et PETIT'S fluid, *Brebissonia*, 1880, p. 92; CARNOY'S *Biol. cell.*, p. 95).

Camphor water (not saturated) .	75 grms.
Distilled water	75 „
Crystallised acetic acid . . .	1 grm.
Acetate of copper	0·30 „
Chloride of copper	0·30 „

This is certainly a most valuable medium for work with delicate fresh tissues. It may be used in combination with

methyl green, which it does not precipitate. The most delicate elements are perfectly preserved in it; the addition of a drop of osmic acid or corrosive sublimate does not cause the least turbidity, and enhances its *fixing* action.

387. Tannin (Carnoy, l. c.).

Water	100 grms.
Powdered tannin	0·50 grm.

388. Picro-carmine.—Picro-carmine has been recommended by Ranvier as a medium for teasing fresh tissues in, in the belief that it possesses sufficient fixing action to preserve the form of cells. Carnoy finds that cells live in it for a considerable time, and become gorged with water and deteriorated to a considerable degree. Unfortunately, too, picro-carmine cannot be combined with a good fixing agent, as it is precipitated by alcohol and by acids, and especially by osmic acid.

389. Methyl Green.—See under Staining Agents. The aqueous solution is sometimes very useful as an examination medium for fresh tissues. It should be taken fairly concentrated, in which state it has sufficient fixing power, which is enhanced by the addition of a trace of osmic acid.

390. Wickersheimer's Fluid (*Zool. Anz.*, 1879, p. 670; cf. *Journ. Roy. Mic. Soc.*, 1882, p. 427; id., 1880, p. 355; and *Entomol. Nachr.*, 1880, p. 129).—This once famous fluid appears to be quite unsuccessful for histological purposes.

391. Meyer's Salicylic Vinegar Preservative Solutions (*Arch. mik. Anat.*, xiii, 1876, p. 868).—"Salicylic vinegar" is a solution of 1 part of salicylic acid in 100 parts of pyroligneous acid. The pyroligneous acid should be of 1·04 specific gravity, and should be of a pale yellow colour. This product is found in commerce, and may be obtained from Herrn J. M. Andreæ, Droguerie-Handlung, Frankfurt-a.-M.

1st Fluid:

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin, viz. glycerin 1 vol., water 2 vols.

For various larvæ, Hydræ, Nematodes, &c.

2nd Fluid:

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin, viz. glycerin 1 vol., water 4 vols.

For Infusoria.

392. Noll's Salicylic Vinegar and Gum Medium (*Zool. Anz.*, 1883, p. 472).—A mixture of equal vols. of Meyer's second fluid (*ante*, last formula) and Farrant's medium (*post*, 395).

This mixture never becomes turbid, and does not dry up. The covers may be luted with asphalt or any other cement. The fluid answers admirably for delicate Crustacea and their larvæ; the preparations do not shrink, and are not too much cleared. It also answers well for hardened and stained preparations of Hydroids, small Medusæ, and other Cœlenterates.

393. Dean's Medium (see *Micro. Dict.*, art. "Preservation").—Appears to be now superfluous.

394. Hoyer's Gum with Chloral Hydrate or Acetate of Potash (*Biol. Centralb.*, ii, 1882, pp. 23–4; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, pp. 144–5).—A high 60 c.c. glass with a wide neck is filled two thirds full with gum arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin is added, *or* acetate of potash or ammonia. The gum with frequent shaking dissolves in a few days, and forms a syrupy fluid, which is slowly filtered for twenty-four hours. The clear filtered fluid will keep a long time, but if spores of fungi begin to develop a little chloral can be added and the fluid refiltered. The solution with chloral is for carmine or hæmatoxylin objects—that with acetate for anilin objects.

395. Gum and Glycerin Medium (Farrant's medium; Beale, *How to Work*, &c., p. 58).

Picked gum arabic	4 ounces.
Water	4 ,,
Glycerin	2 ,,

To be kept in a stoppered bottle with a lump of camphor.

This medium is quoted by Frey as consisting of equal parts of gum, glycerin, and saturated aqueous solution of arsenious acid.

The *Micrographic Dictionary* gives the following directions:

Gum arabic 1 ounce, glycerin 1 ounce, water 1 ounce, arsenious acid 1½ grains; dissolve the arsenious acid in the water, then the gum (without heat), add the glycerin, and incorporate with great care to avoid forming bubbles.

Another method for making this medium is given by A. F. Stanley Kent in *Journ. Roy. Mic. Soc.*, 1890, p. 820.

396. Gum and Glycerin Medium (Langerhans' formula,

modification of FARRANT'S medium, *Zool. Anzeig.*, ii, 1879, p. 575).

Gummi arab.	5·0
Aquæ	5·0

To which after twelve hours are added—

Glycerini	5·0
Sol. aquosa acid. carbol. (5·100) . .	10·0

Marine animals may be preserved in this by simply running in a drop under the cover, and next day or later adding what is necessary to make up for evaporation, and closing the mount. Shrinkage is very slight, and most colours keep well.

397. FARIS'S **Glycero-Gum** (*The Microscope*, x, 1890, p. 59; *Journ. Roy. Mic. Soc.*, 1890, p. 414).—Gum arabic, 2 ounces; glycerin, 1·5 ounces; water, 1·5 ounces; thymol, 1 grm. Mix, dissolve with heat, and filter.

398. Gum and Glycerin Jelly (SHIMER, *The Microscope*, ix, 1889, p. 138; *Journ. Roy. Mic. Soc.*, 1890, p. 411).—Equal parts of glycerin jelly (FOL'S second formula, *post*, § 412), Farrant's solution, and glycerin.

399. COLE'S **Gum and Syrup Medium** (see above, § 317).

400. APÁTHY'S **Gum and Syrup Medium** (see above, Chap. IX, § 119).—This medium is recommended by Apáthy in a general way, and not merely for the special purpose for which it is quoted in § 119. It sets very hard, and, combined with a paper cell (see Chap. XX), may be used for ringing glycerin mounts.

401. Fabre-Domergue's Glucose Medium (*La Nature*, No. 823, 9 Mars, 1889, supp.).

Glucose syrup diluted to twenty-five degrees of the areometer (sp. gr. 1·1968) . . .	1000 parts.
Methyl alcohol	200 „
Glycerin	100 „
Camphor, to saturation.	

The glucose is to be dissolved in warm water, and the other ingredients added. The mixture, which is always acid, must be neutralised by the addition of a little potash or soda.

This medium is said to preserve without change almost all

animal pigments. If it really performs this, its great value is evident.

402. Brun's **Glucose Medium** (from Fabre-Domergue's *Premiers Principes du Microscope et de la Technique microscopique*, Paris, 1889, p. 123).

Distilled water	140 parts.
Camphorated spirit . . .	10 „
Glucose	40 „
Glycerin	10 „

Mix the water, glucose, and glycerin, then add the spirit, and filter to remove the excess of camphor which is precipitated on mixing. I am indebted to Dr. Henneguy for calling my attention to this liquid, which is an important one. It is preferable to glycerin because it preserves the colour of preparations stained with anilin dyes, *methyl green included.*

402a. Levulose as a Mounting Medium.—Levulose is recommended as a mounting medium by Behrens, Kossel, u. Schiefferdecker (*Das Mikroskop. u. d. Meth. d. mik. Unters.*, Braunschweig, 1889). It is uncrystallisable, and preserves well carmine and coal-tar stains (hæmatoxylin stains fade somewhat in it). The index of refraction is somewhat higher than that of glycerin. Objects may be brought into it out of water.

Glycerin Media.

403. Glycerin.—Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable from an optical point of view, on account of the increased visibility that it gives to many structures by lowering the index of refraction of the glycerin. But from the point of view of efficacious preservation it is always advisable to use undiluted glycerin, the strongest that can be procured.

Long soaking of tissues in glycerin of gradually increased strength is a necessary preliminary to mounting in all cases in which it is desired to obtain the best possible preparations, and to ensure that they shall keep well. If this soaking is done on the slide (the cover being removed and the object treated with fresh glycerin every one or two days), it is well to take the precaution recommended by Beale, of luting the edges of the cover so as to make the preparation air-tight, as glycerin is so highly hygroscopic that a drop of it exposed to the air rapidly diminishes in strength to a very considerable

degree. In order to facilitate the removal of the cover in this process, the slide may be gently warmed by passing it two or three times through the flame of a spirit lamp. No preparation can be considered to be made *secundum artem* until every part of the object has been thoroughly impregnated with strong pure glycerin.

The shrinking that frequently occurs when delicate structures are brought into glycerin may generally be cured by this treatment; cells which at first appear hopelessly collapsed gradually swell out to their normal forms and dimensions.

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin jelly;* as soon as this is set a coat of any of the usual cements may be applied. This has of course been for the last twenty years one of the commonplaces of histological technic, but that has not prevented somebody from recently describing the process at great length as new.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

The already high index of refraction of glycerin (Price's glycerin, $n = 1{\cdot}46$) may be raised to about that of crown glass by dissolving suitable substances in the glycerin. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$)* in glycerin may be 1·504; that of a saturated solution of sulphocarbonate of zinc in glycerin may be 1·501; that of a saturated solution of Schering's† chloral hydrate (in crusts) in glycerin is 1·510; that of iodate of zinc in glycerin may be brought up to 1·56.‡ The clearing action of glycerin may thus be greatly increased, and the full aperture of homogeneous objectives brought to bear on objects mounted in one of the above-named solutions.

The sulphocarbolate of zinc solution§ may be prepared by taking equal parts by weight of Price's glycerin and sulphocarbolate of zinc crystals, mingling the two, and applying sufficient heat to boil the glycerin. The solution can be made in about an hour, but no fear need be had about boiling too long, as the longer this is done the less liability will there be for the solution to deposit crystals on the bottom of the bottle when cooled, which it will do if the temperature is only kept up long enough to dissolve the

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* *Journ. Roy. Mic. Soc.*, ii, 1879, p. 346.

† *Ibid.* (N.S.), i, 1881, p. 943.

‡ *Ibid.*, p. 366.

§ *Ibid.*, iii, 1880, p. 1051.

crystals. Filter while hot. The index may be brought up to 1·525 if desired, by evaporating the solution somewhat, or by adding more carbolate.

404. Barff's Boroglyceride (see *Journ. Roy. Mic. Soc.*, 1882, p. 124).—This preparation may be obtained (price 1*s.* per bottle) from *The Kreochyle Company, Viaduct House, Farringdon Street, E.C.*, or all wholesale chemists.

405. Glycerin and Alcohol Mixtures.—These most useful fluids afford one of the best means of bringing delicate objects gradually from weak into strong glycerin. The object is mounted in a drop of the liquid, and left for a few hours or days, the mount not being closed. By the evaporation of the alcohol the liquid gradually increases in density, and after some time the mount may be closed, or the object brought into pure glycerin or glycerin jelly.

1. Calberla's Liquid:

Glycerin	1 part.
Alcohol	1 „
Water	1 „

A most valuable examination fluid. As already pointed out (§ 2), this liquid is in many cases to be preferred to alcohol for keeping fixed objects in until required for dissection or other further preparation.

2. I strongly recommend the following for very delicate objects:

Glycerin	1 part.
Alcohol	1 „
Water	2 parts.

3. Hæntsch's Liquid:

Glycerin	1 part.
Alcohol	3 parts.
Water	2 „

4. Jäger's Liquid (quoted from Vogt and Yung's *Traité d'Anat. comp. prat.*, p. 16):

Glycerin	1 part.
Alcohol	1 „
Sea water	10 parts.

406. Deane's Glycerin Jelly (from Frey's *Le Microscope*, p. 231).—120 grammes glycerin, 60 grammes water, 30 grammes gelatin. Dissolve the gelatin in the water, and add the gly-

cerin. This, and the following glycerin jellies, must of course be used warm.

407. Lawrence's Glycerin Jelly (DAVIES, *Preparation and Mounting of Microscopic Objects*, p. 84).—"He takes a quantity of Nelson's gelatin, soaks it for two or three hours in cold water, pours off the superfluous water, and heats the soaked gelatin until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool*, he adds a fluid drachm of the white of an egg. He then boils this until the albumen coagulates and the gelatin is quite clear, when it is to be filtered through fine flannel, and to each ounce of the clarified solution add 6 drachms of a mixture composed of 1 part of glycerin to 2 parts of camphor water."

408. Beale's Glycerin Jelly (*How to Work*, &c., p. 57).—Gelatin or isinglass, soaked, melted, and clarified if desired, as in the last formula. To the clear solution add an equal bulk of strong glycerin.

409. Brandt's Glycerin Jelly (*Zeit. f. Mik.*, ii, 1880, p. 69; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 502).—Melted gelatin 1 part, glycerin 1½ parts.

The gelatin to be soaked in water and melted in the usual way. After incorporating the glycerin, the mixture is to be filtered. This is a point of vital importance, as the gelatin of commerce is always mixed with particles of dust and minute threads. Swedish filtering-paper does not allow the fluid to pass through sufficiently, and flannel produces more threads than before. The following simple apparatus is found effective. A wide-necked bottle is broken in two, and the upper part taken. The neck is stopped with a cork having two holes bored in it. In the first hole a glass tube, about 20 cm. long, is inserted so as to project a little into the inside of the bottle, and on the outside it is bent sharply to one side and drawn out into a point of about 1½ to 2 mm. diameter. In the second hole a funnel-shaped filter is inserted so that the conical part is inside the bottle and the tube projects a few centimetres beyond the cork and the neck of the bottle. The apparatus is then placed so that the wide opening of the bottle and of the funnel is uppermost, and some spun glass is pressed into the lower conical part of the filter. In using

the apparatus the funnel is filled with glycerin gelatin, and the bottle with hot water, which runs off slowly through the tube in the first hole and is constantly replenished.

Some drops of carbolic acid should be added to the fluid product of the filtering. For mounting, use warm by melting a small portion on the slide, the object having been previously soaked for some time in a small bottle of the medium warmed with a suitable apparatus.

410. Kaiser's Glycerin Jelly (*Bot. Cent.*, i, 1880, p. 25; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 504).—One part by weight finest French gelatin is left for two hours in 6 parts by weight distilled water, 7 parts of glycerin are added, and for every 100 grammes of the mixture 1 gramme of concentrated carbolic acid. Warm for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass laid wet in the filter. Use for mounting as above.

I prepared some of this jelly many years ago, and find it is still perfectly clear.

411. Seaman's Glycerin Jelly (*Amer. Mon. Mic. Journ.*, ii, 1881, p. 45; *Journ. Roy. Mic. Soc.* [N.S.], i, 1881, p. 534).—Dissolve isinglass in water, so that it makes a stiff jelly when at the ordinary temperature of the room, add one tenth as much glycerin, and a little solution of borax, carbolic acid, or camphor water. Filter whilst warm through muslin, and add a little alcohol.

412. Fol's Glycerin Jellies (*Lehrb.*, p. 138).

1. Melt together one volume of Beale's jelly (§ 408) and one half to one volume of water, and add 2 to 5 per cent. of salicylic acid solution, or carbolic acid or camphor.

2.	Gelatin	30 parts.
	Water	70 „
	Glycerin	100 „
	Alcoholic solution of camphor . .	5 „

Prepare as before, adding the camphor last.

3.	Gelatin	20 parts.
	Water	150 „
	Glycerin	100 „
	Alcoholic solution of camphor . .	15 „

413. Squire's **Glycerin Jelly** (Squire's *Methods and Formulæ*, &c., p. 84).—Soak 100 grms. of French gelatin in chloroform water, drain when soft, and dissolve with heat in 750 grms. of glycerin. Add 400 grms. of chloroform water with which has been incorporated about 50 grms. of fresh egg-albumen; mix thoroughly, and heat to boiling-point for about five minutes. Make up the total weight to 1550 grms. with chloroform water. Filter in a warm chamber.

414. Stephenson's Biniodide of Mercury and Iodide of Potassium (*Journ. Roy. Mic. Soc.*, 1882, p. 167).—Interesting as giving a solution which when saturated has an index of 1·680, the highest index of any known aqueous fluid. I have experimented both with strong and weak solutions, and doubt whether much practical advantage can be derived from them. Tissues are well preserved, but the preparations are ruined by a precipitate which forms in the fluid.

415. Monobromide of Naphthalin.—See *Journ. Roy. Mic. Soc.*, 1880, p. 1043 (Abbe and van Heurck), and *Zool. Anz.*, 1882, p. 555 (Max Flesch).

416. Thompson's **High Refractive Medium**,—See *Journ. Roy. Mic. Soc.*, 1892, p. 902.

Resinous Media.

417. Resins and Balsams.—Resins and balsams consist of a vitreous or amorphous substance held in solution by an essential oil. By distillation or drying in the air they lose the essential oil and pass into the solid state. It is these solidified resins that should, in my opinion (and that, I believe, of the best microscopists), be employed for microscopical purposes; for the raw resins always contain a certain proportion of water, which makes it difficult to obtain a clear solution with the usual menstrua, is injurious to the optical properties of the medium and to its preservative qualities, and, further, especially hurtful to the preservation of stains. I therefore recommend that all solutions be made by heating gently the balsam or resin in a stove until it becomes brittle when cold, and then dissolving in an appropriate menstruum.

Fol (*Lehrb.*, pp. 138–9) is of a different opinion.

Solutions made with volatile menstrua, such as xylol and chloroform, set rapidly, but become rapidly brittle. Solutions made with non-volatile media, such as turpentine, set much less rapidly, and pass much less rapidly into the brittle state.

As to the old dispute about the respective merits of damar and balsam, the case appears, after all, to lie in a nutshell. Damar gives the better definition of delicate detail; balsam has greater clearing action, and affords perhaps more solid mounts.

It may be remarked here that for some of the purposes for which these media are employed, **Oil of Cedar** may be found preferable. The mounts need not be closed (except for immersion work), as the oil soon sets hard enough to keep the cover in place.

418. Canada Balsam.—Prepare with the solid balsam as above described. The usual menstrua are xylol, benzol, chloroform, and turpentine. Dissolve the solid balsam in one of these to the required consistence. The turpentine solution is to be preferred only in cases where it is desired to have a medium that sets very slowly, or in view of the better preservation of certain stains. For most other purposes the xylol solution is the best. If time be an object, a benzol solution should be preferred, as it sets much quicker than the xylol solution.

Heys states that if the chloroform solution be poured into long, thin, half-ounce phials, corked up, and set aside for at least a month, the medium will be clearer and set much quicker than if the balsam is mixed with the chloroform at the time it is required for use (*Trans. Mic. Soc.*, Jan., 1865, p. 19; Beale, p. 51).

Sahli (*Zeit. f. wiss. Mik.*, 1885, p. 5) recommends *oil of cedar* as a menstruum.

419. Seiler's Alcohol Balsam (*Proc. Amer. Soc. Mic.*, 1881, pp. 60–2; *Journ. Roy. Mik. Soc.* [N.S.], ii, 1882, pp. 126–7).—"Take a clear sample of Canada balsam and evaporate it in a water- or sand-bath to dryness; *i. e.* until it becomes brittle and resinous when cold. Dissolve this while warm in warm absolute alcohol and filter through absorbent cotton."

The advantage of this medium is stated to be that objects may be mounted in it direct from absolute alcohol, without previous treatment with an essential oil or or other clearing agent; Seiler considers that by this means "shrivelling is avoided, as well as *the solution of fat in the cells.*"

The process is not very easy to carry out, and I cannot recommend it for general work.

420. Damar (Gum Damar, or Dammar, or d'Ammar).—The menstrua are the same as for balsam, and the solution should

be prepared in the same way. The most beautiful of all these mounting media is the solution of damar in xylol. Heat is not necessary to make the solution.

Minute directions (which I think unnecessary) for preparing a working solution are given by MARTINOTTI in *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 156, and in *Malpighia*, ii, 1888, p. 270; cf. also *Journ. Roy. Mic. Soc.*, 1889, p. 163.

FLEMMING, PFITZNER, and a writer signing C. J. M., all employ a mixture of benzol and turpentine (see *Arch. mik. Anat.*, xix, 1881, p. 322; *Sci. Gossip*, 1882, p. 257; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 145; *Morphol. Jahrb.*, vi, 1880, p. 469; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 583).

MAX FLESCH notes hereon (*Zool. Jahresber. f.* 1880, p. 51) that at Würzburg the ordinary dammar varnish of arists is employed.

JAMES (*Engl. Mech.*, 1887, p. 184; *Journ. Roy. Mic. Soc.*, 1887, p. 1061) also gives some, I think, superfluous formulæ for damar solutions; and still another new method is given, op. cit., 1890, p. 680.

A formula for a damar and mastic medium is given by SQUIRE, in his *Methods and Formulæ*, &c., p. 84.

421. Balsam-Damar.—It has been recommended to mix equal volumes of benzol balsam and turpentine damar, but this medium seems to me superfluous.

422. Colophonium.—A solution of colophonium in turpentine has been recommended by Kleinenberg. I find it works very pleasantly. The palest kind of colophonium should of course be taken.

This medium sets very slowly, so that ample time is afforded for arranging objects in it. Kleinenberg warns against the employment of absolute alcohol as a solvent; the preparations are beautiful at first, but soon become spoiled by the precipitation of crystals or of an amorphous substance.

The turpentine solution keeps perfectly limpid, gives very good definition, and is altogether so excellent a medium that I am surprised that it is not more used. It should be recommended to beginners.

423. Venice Turpentine for Mounting (VOSSELER, *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 292, *et seq.*).—Vosseler strongly recommends this medium as having considerable advantages over Canada balsam or damar.

Commercial Venice turpentine is mixed in a tall cylinder glass with an equal volume of 96 per cent. alcohol, allowed to stand in a warm place for three or four weeks, and decanted. It is stated that preparations may be mounted in this medium without previous clearing with essential oils or the like. The index of refraction being lower than that of the above-named balsams, delicate details are more distinctly brought out. Stains keep well in the medium, and Vosseler states that he possesses preparations made fifteen years ago that are perfectly well preserved.

This medium is also recommended by SUCHANNEK (*ibid.*, vii, 4, 1891, p. 463). He advises that it be prepared with equal parts of Venice turpentine and neutral absolute alcohol (obtained by treating commercial absolute alcohol with calcined cupric sulphate and quicklime). The mixture should be agitated frequently and kept in a tile stove for a day or two until clear and sufficiently inspissated.

424. Copal Varnish.—I have seen tissues very instructively mounted in this medium, which is probably worthy of further study. "Berry's Hard Finish," which is an easily obtainable copal varnish, has been highly praised for mounting purposes (see *Journ. Roy. Mic. Soc.*, 1887, p. 1064).

425. Castor Oil.—This has been lately recommended as a mounting medium for certain delicate tissues (sections of eyes of Cephalopods) by GRENACHER (*Abhandl. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244). This was with the idea that its low refractive index ($n = 1{\cdot}49$, whilst Canada balsam $n = 1{\cdot}54$) would give a useful augmentation of visibility for the more refractive elements of the tissues.

With the objects with which I have experimented I have not found this to be the case.

426. Photographic Negative Varnish (for mounting large sections without cover-glasses).—See WEIGERT, *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 209.

427. Styrax and Liquidambar.—See *Journ. Roy. Mic. Soc.*, 1883, p. 741; ib., 1884, pp. 318, 475, 655, and 827; and the places there quoted. Also *Bull. Soc. Belge de Mic.*, 1884, p. 178; and FOL, *Lehrb.*, p. 141.

428. Tolu Balsam (see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 471).

429. Frankincense (see *Journ. Roy. Mic. Soc.*, 1892, p. 901).

CHAPTER XX.

CEMENTS AND VARNISHES.

430. Thanks to the efforts of the dilettanti to outshine one another with neatly gaudy "rings," microscopical literature contains a goodly show of receipts for cements and varnishes. I have collected such as appear likely to be useful, rejecting all that relates merely to ornament.

Two, or at most three, of the media given below will certainly be found sufficient for all useful purposes. For many years I have used only one cement (Bell's). I recommend this as a cement and varnish; gold size may be found useful for turning cells; and Miller's caoutchouc cement may be kept for occasions on which the utmost solidity is required.

Marine glue is necessary for making glass cells.

Carpenter lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles;* he has always found that those that do, although they might stand well for a few weeks or months, yet always became porous after a greater lapse of time, allowing the evaporation of the liquid and the admission of air. All fluid mounts *should be ringed with glycerin jelly before applying a cement; by this means all danger of running-in is done away with.*

The above passage stands as it stood, italicised as here, in the 1st and 2nd editions. It was translated and amplified, in a special paragraph, in the *Traité des Méth. techniques.* I may therefore be excused from hunting up the name of the anatomist who recently published as new this old, old method, or the pages of the journals which reproduced his paper without protest.

The reader who requires more information concerning microscopical cements and varnishes than can be given in this chapter may consult with advantage the papers of Aubert, *The Microscope,* xi, 1891, p. 150, and *Journ. Roy.*

Mic. Soc., 1891, p. 692; BECK, *The Microscope*, xi, 1891, pp. 338, 368, and *Journ. Roy. Mic. Soc.*, 1892, p. 293; and the last edition of BEHRENS' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten* (Bruhn, Braunschweig, 1892).

431. Gelatin Cement (MARSH'S *Section-cutting*, 2nd ed., p. 104).—Take half an ounce of Nelson's opaque gelatin, soak well in water, melt in the usual way, stir in 3 drops of kreasote, and put away in a small bottle. It is used warm.

When the ring of gelatin has become quite set and dry, which will not take long, it may be painted over with a solution of bichromate of potash made by dissolving 10 grains of the salt in an ounce of water. This should be done in the daytime, as the action of daylight is necessary to enable the bichromate to render the gelatin insoluble in water. The cover may then be finished with Bell's cement.

This process is particularly adapted for glycerin mounts.

432. The Paper Cell Method.—According to my experience, the best way to make a fluid mount safe is the following:—By means of two punches I cut out rings of paper of about a millimetre in breadth, and of about a millimetre smaller in diameter than the cover-glass. *Moisten* the paper ring with mounting fluid, and centre it on the slide. Fill the cell thus formed with mounting fluid; arrange the object in it; put the cover on; fill the annular space between the paper and the margin of the cover with glycerin jelly (a turn-table may be useful for this operation); and as soon as the gelatin has set turn a ring of Bell's or other cement on it.

For greater safety, the gelatin may of course be treated with bichromate according to Marsh's plan.

433. Comparative Tenacity of Cements (see BEHRENS, *Zeit. f. wiss. Mik.*, ii, 1885, p. 54, and AUBERT, *Amer. Mon. Mic. Journ.*, 1885, p. 227; *Journ. Roy. Mic. Soc.*, 1886, p. 173).—Behrens gives the palm to amber varnish; Aubert places Miller's caoutchouc cement at the head of the list, Lovett's cement coming halfway down, and zinc white cement at the bottom, with less than one quarter the tenacity of the caoutchouc cement.

434. Bell's Cement.—Composition unknown. May be ob-

tained from the opticians or from J. Bell and Co., chemists, 338, Oxford Street, London.

This varnish flows easily from the brush, and sets quickly. For glycerin or other fluid mounts, the cover should be ringed as above described with glycerin jelly before applying the varnish. This precaution is especially necessary with glycerin. This is the best varnish for fluid mounts known to me. It is soluble in ether or chloroform. It is not attacked by oil of cedar.

435. Miller's Caoutchouc Cement.—Composition unknown. May be obtained from the opticians. A very tenacious and, which is frequently an important point, a quickly drying cement.

436. Asphalt Varnish (*Bitume de Judée*).—Unquestionably one of the best of these media, either as a cement or a varnish *provided it be procured of good quality.* It can be procured from the opticians or from the oil-shops. KITTON (*Month. Mic. Journ.*, 1874, p. 34) recommends asphalt dissolved in benzol with the addition of a small quantity of gold size.

437. Brunswick Black.—Best obtained from the opticians. A receipt for preparing it is given in BEALE, *How to Work*, &c., p. 49.

"If a little solution of india rubber in mineral naphtha be added to it, there is no danger of the cement cracking when dry." Carpenter states that without this addition it is brittle when dry. Brunswick black is soluble in oil of turpentine. A most useful cement, works easily and dries quickly. It can be recommended for turning cells.

438. Brunswick Black and Gold Size (EULENSTEIN, BEALE, *How to Work*, &c., p. 49).—Equal parts of Brunswick black and gold size with a very little Canada balsam.

439. Gold Size.—Receipts for preparing it may be found in the *Micrographic Dict.* or in COOLEY'S *Cyclopædia;* but it is certainly best to obtain it from the opticians or oil-shops. It is soluble in oil of turpentine. A good cement, *when of good quality*, and very useful for turning cells.

440. Marine Glue.—Found in commerce. Carpenter says the best is that known as G K 4.

It is soluble in ether, naphtha, or solution of potash. Its

use is for attaching glass cells to slides, and for all cases in which it is desired to cement glass to glass.

Receipts for preparing it may be found in BEALE, p. 49, or in COOLEY'S *Cyclopædia.*

441. Harting's Gutta-percha Cement (see BEALE'S *How to Work,* &c., p. 49).—Marine glue serves the same purpose, viz. that of attaching cells to slides.

442. India-rubber and Lime French Cement.—See BEALE, p. 58.

443. Knotting (*Journ. Roy. Mic. Soc.,* 1882, p. 745).—"Patent knotting" from oil and colour stores, exposed to the air until it has become of the proper consistency;—for mending cells and for preventing running-in of the finishing varnish (*Northern Microscopist,* ii, 1882).

444. Turpentine, Venice Turpentine (CSOKOR, *Arch. mik. Anat.,* xxi, 1882, p. 353; PARKER, *Amer. Mon. Mic. Journ.,* ii, 1881, pp. 229–30; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 724).—Venice turpentine (Terebinthina veneta) is the liquid resinous exudation of *Abies larix.* It is seldom met with in a pure state. The following are the directions for preparing and using it given by Parker:

Dissolve true Venice turpentine in enough alcohol, so that after solution it will pass readily through a filter, and, after filtering, place in an evaporating dish, and by means of a sand-bath evaporate down to about three quarters of the quantity originally used. (The best way to tell when the evaporation has gone far enough is to drop some of the melted turpentine, after it is evaporated down to about three quarters its original volume, into cold water; if on being taken out of the water it is hard and breaks with a vitreous fracture on being struck with the point of a knife, cease evaporation and allow to cool.)

Or (CSOKOR), common resinous turpentine of commerce is put in small pieces to melt over a water-bath, then poured into a suitable vessel and allowed to cool. It should form a brittle, dark brown mass, not yielding to the pressure of a finger. It is sometimes useful, in order to attain the right degree of hardness in the cold mass, to add a little resinous

oil of turpentine to the melted mass, and then to evaporate for several hours over the water-bath.

This cement is used for closing glycerin mounts; it is applied in the following manner:—Square covers are used, and superfluous glycerin is cleaned away from the edges in the usual way.

The cement is then put on with a piece of wire bent at right angles (No. 10—12 wire is taken, and copper is the best, as it gives to the turpentine a greenish tinge); the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit lamp, plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure, very handy, and never runs in. Parker saw this cement, or a similar one known as *Venedischer Damarlack*, exclusively used for glycerin mounts in the Pathological Laboratory at Vienna.

This is an extremely valuable method. It is very rapid and very safe. The cement sets hard in a few seconds.

445. Colophonium and Wax (Krönig, *Arch. f. mik. Anat.*, 1886, p. 657; *Journ. Roy. Mic. Soc.*, 1887, p. 344).—Seven to nine parts of colophonium are added piecemeal to two parts of melted wax, the whole filtered and left to cool. For use, the mass is melted by placing the containing vessel in hot water. The cement is not attacked by water, glycerin, or caustic potash.

446. Amber Varnish.—As above mentioned, Behrens finds this cement to possess an extreme tenacity. He does not give the composition of his varnish, which was procured from E. Pfannenschmidt at Dantzic. The following is from Cooley's *Cyclopœdia*, art. "Varnish:"

"Take of amber (clear and pale) 6 lbs., fuse it; add of hot clarified linseed oil 2 gallons, boil it until it "strings" well, then let it cool a little and add of oil of turpentine 4 gallons or q. s."

Other receipts, l. c.

447. Amber and Copal Varnish (HEYDENREICH, *Zeit. f. wiss. Mik.*, 1885, p. 338).—An extremely complicated mode of preparation. The varnish may be obtained from Ludwig Marx, at 110, Moskowskaja Sastawa, St. Petersburg; or 79, Gaden, Vienna; or 1, Römerthal, Mayence.

448. Shellac Varnish (BEALE, p. 28).—Shellac should be broken into small pieces, placed in a bottle with spirit of wine, and frequently shaken until a thick solution is obtained. The *Micro. Dictionary* says that the addition of 20 drops of castor oil to the ounce is an improvement.

Untrustworthy, but useful for protecting balsam mounts from the action of oil of cedar.

For a method of preparing chemically pure shellac (a somewhat important matter), see WITT, *Zeit. f. wiss. Mik.*, 1886, p. 199.

For SEAMAN'S shellac cement for attaching metal to glass, see *Journ. Roy. Mic. Soc.*, 1888, p. 520.

449. Sealing-wax Varnish (*Micro. Dict.*, "Cements").—Add enough spirit of wine to cover coarsely powdered sealing-wax, and digest at a gentle heat. This should only be used as a varnish, never as a cement, as it is apt to become brittle and to lose its hold upon glass after a time.

450. Tolu Balsam Cement (CARNOY'S *Biol. Cell.*, p. 129).

Tolu balsam	2 parts.
Canada balsam	1 part.
Saturated solution of shellac in chloroform	2 parts.

Add enough chloroform to bring the mixture to a syrupy consistence. Carnoy finds this cement superior to all others.

451. Stieda's White Zinc Cement (*Arch. f. mik. Anat.*, 1866, p. 435).—Rub up oxide of zinc with turpentine, and add, stirring continually, for every drachm of the zinc oxide 1 ounce of a solution of damar in turpentine (of the consistency of thick syrup). This gives a *white* cement like Ziegler's. For a red cement take, instead of zinc, *cinnabar*, and take 2 drms. of the metal for each ounce of the damar solution. If the cement has become too thick with age, dilute with turpentine, ether, or chloroform.

452. Ziegler's White Cement.—Composition unknown. Is very much used on the Continent.

453. Kitton's White-lead Cement (*Month. Mic. Journ.*, 1876, p. 221).—Equal parts of white-lead, red-lead, and litharge (all in powder), ground together with a little turpentine until thoroughly incorporated, then mixed with gold size. The mixture should be thin enough to work with a brush. No more of the cement should be made than is required for present use, as it soon sets and becomes unworkable; but a stock of the materials may be kept ready ground in a bottle.

454. Lovett's Cement (*Journ. Roy. Mic. Soc.*, 1883, p. 786).—Two parts white-lead, 2 parts red oxide of lead (minium), 3 parts litharge. To be ground very fine, mixed dry, and kept so in a bottle. When required for use mix a little of the powder with gold size to the consistency of paint, taking care that no grit gets into it.

455. Apáthy's Cement for Glycerin Mounts (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 171).—Equal parts of hard (60° C. melting-point) paraffin and Canada balsam. Heat together in a porcelain capsule until the mass takes on a golden tint, and no longer emits vapours of turpentine. On cooling this forms a hard mass, which is used by warming and applying with a glass rod or brass spatula. One application is enough. The cement does not run in, and never cracks.

456. Aspinall's Enamel.—Stanley Kent (*Journ. Roy. Mic. Soc.*, 1890, p. 821) finds this of great use, both for ringing slides and making cells.

CHAPTER XXI.

INJECTIONS—GELATIN MASSES.

457. Introduction.—Injection masses are composed of a coloured substance, technically termed the *colouring mass*, and of a substance with which that is combined, technically termed the *vehicle*.

The following formulæ are grouped according to the nature of the vehicle. A note on the employment of **nitrite of amyl** for provoking the dilatation of vessels will be found at § 500.

458. Robin's Gelatin Vehicle (*Traité*, p. 30).—Take some gelatin, of the sort known as "colle de Paris." (This gelatin is found in commerce in the form of thin sheets, marked with lozenge-shaped impressions of the cords which supported them whilst drying.) Soak it in cold water, then heat in water over a water-bath. One part of gelatin should be taken for every 7, 8, 9, or even 10 parts of water; it is a common error to employ solutions containing too much gelatin. The solution is now to be combined with one of the colouring masses given below.

This vehicle, like all gelatin masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve it.

Chloral hydrate added to the mass will preserve it (HOYER). A sufficient dose, at least 2 per cent., should be employed (see below, §§ 472, 473).

459. Robin's Glycerin-Gelatin Vehicle (*Traité*, p. 32).—Dissolve in a water-bath 50 grms. of French gelatin ("colle de Paris") in 300 grms. of water in which has been dissolved some arsenious acid; add of glycerin 150 grms., and of carbolic acid a few drops. Unlike the pure gelatin vehicles, this mass does keep indefinitely.

The colouring masses recommended for combination with the vehicles above described are made as follows:

460. Carmine Colouring Mass (*Traité*, p. 33).—Rub up in a mortar 3 grms. of carmine with a little water and enough ammonia to dissolve the carmine. Add 50 grms. of glycerin, and filter.

Prepare 50 grms. of acid glycerin (containing 5 grms. of acetic acid for every 50 grms. of glycerin), and add it by degrees to the carmine-glycerin, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the gelatin injection vehicle (*ante*, Formula 458), or of the glycerin-gelatin (No. 459), or glycerin-alcohol vehicle described below (No. 502).

461. Ferrocyanide of Copper Colouring Mass (*ibid.*, p. 34).

Take—

(1)	Ferrocyanide of potassium (concentrated solution)	20 c.c.
	Glycerin	50 „
(2)	Sulphate of copper (concentrated solution)	35 „
	Glycerin	50 „

Mix (1) and (2) slowly, with agitation; at the moment of injecting combine with 3 volumes of vehicle.

462. Blue Colouring Mass (Prussian Blue) (*Robin's modification of Beale's formula, ibid.*, p. 35).

Take—

(A)	Sulphocyanide of potassium (sol. sat.)	90 c.c.
	Glycerin	50 „
(B)	Liquid perchloride of iron at 30°	3 „
	Glycerin	50 „

Mix slowly and combine the mixture with 3 parts of vehicle. It is well to add a few drops of HCl.

463. Cadmium Colouring Mass (*ibid.*, p. 36).

Take—

Sulphate of cadmium (sol. sat.)	40 c.c.
Glycerin	50 „
and	
Sulphide of sodium (sol. sat.)	30 „
Glycerin	50 „

Mix with agitation and combine with 3 vols. of vehicle.

464. Scheele's Green Colouring Mass (*ibid.*, p. 37).

Take—

Arseniate of potash (saturated solution)	80	c.c.
Glycerin	50	,,

and

Sulphate of copper (saturated solution)	40	,,
Glycerin	50	,,

Mix and combine with 3 vols. of vehicle.

465. Anilin Colouring Masses (*ibid.*, p. 37).—Most of the anilin colours have, for injections, the great fault of being soluble in alcohol; fuchsin is soluble in water, in alcohol, and in glycerin; it therefore cannot be employed with a gelatin or glycerin vehicle. Anilin blue, violet, yellow, may be combined with these vehicles after dissolving in a small quantity of alcohol; and (alcohol being avoided for hardening purposes) the injected organs may be preserved in glycerin.

Carmine-Gelatin Masses.

466. Ranvier's Carmine-Gelatin Mass (*Traité technique*, p. 116).—Take 5 grms. Paris gelatin, soak it in water for half an hour, or until quite swollen and soft; wash it; drain it; put it into a test-tube and melt it, in the water it has absorbed, over a water-bath. When melted add slowly, and with continual agitation, a solution of carmine in ammonia, prepared as follows:—2½ grms. of carmine are rubbed up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

When the carmine has been added to the gelatin you will have about 15 c.c. of ammoniacal solution of carmine in gelatin, if the operations have been properly performed. This solution is to be kept warm on the water-bath, whilst you proceed to neutralise it by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in 2 parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived, the addition of acetic acid must cease, and the liquid be examined under the microscope. If it contains a

granular precipitate of carmine, too much acid has been added, and the mass must be thrown away.

Ranvier states that by practice the operator learns to attain to perfect neutralisation almost infallibly in this way, and that this is the only way to attain to it. Trust must not be put in certain formulæ that profess to indicate the proportions of ammonia and acetic acid necessary for neutralisation, on account of the variation in strength of the solutions of ammonia kept in laboratories. The method proposed by Frey of determining beforehand the quantity of a known acetic solution that is necessary for neutralisation of a given quantity of the ammonia employed, is not infallible because it often happens that commercial gelatin is acid ; in which case the proposed method would cause the operator to overpass the point of saturation.

The mass having been perfectly neutralised is strained through new flannel.

467. How to Neutralise a Carmine Mass (Ville, *Gaz. hebd. d. Sci. méd. de Montpellier,* Fév., 1882; may be had separately from Delahaye et Lecrosnier, Paris).—Ville is of Ranvier's opinion that the method of titration recommended by Frey is defective, but for a different reason. When carmine is treated with ammonia a certain proportion of the ammonia combines with the carmine to form a transparent purple compound, and the rest of the ammonia remains in excess. It is this *excess* that it is required to neutralise precisely. In Frey's method a quantity of acid sufficient for the neutralisation of the *whole* of the ammonia employed is taken; hence, naturally, the point of neutralisation is overstepped, and a granular mass is the result.

As to the acidity accidentally found in commercial gelatin, that source of error is easily eliminated. Instead of soaking the gelatin in water, it should be placed in a large funnel with a narrow neck, or, better, in a stopcock funnel, and the whole should be placed under a tap, and a stream of water arranged in such a manner that the gelatin be constantly completely immersed. Washing for an hour or so in this way will remove all traces of acids mechanically retained in the gelatin.

As to the neutralisation of the colouring mass, Ville is of

opinion that the criterion of neutrality given by Ranvier—the sour smell that takes the place of the ammoniacal odour—cannot be safely relied on in practice. He considers it greatly preferable to employ dichroic litmus paper (litmus paper sensitized so as to be capable of being used equally for the demonstration of acids and bases).

To prepare such a paper, the tincture obtained by decoction of cake litmus is slightly acidified by an excess of sulphuric acid. By this means the excess of alkali, or of alkaline carbonates, that is always present in litmus decoction, and which diminishes its sensibility as a reagent, is neutralised. The decoction is then heated and agitated with an excess of precipitated carbonate of baryta, and filtered.

The solution of litmus thus obtained is exposed to the air in wide vessels until its intense blue colour has given place to a reddish tint. Strips of white unsized paper are then dipped in it, and dried in the shade on stretched threads, in a place free from vapour of ammonia.

A shorter method consists in adding very dilute sulphuric acid, drop by drop, to the ordinary laboratory tincture of litmus, until the colour changes to red. Then, by adding successively traces of alkali and very dilute sulphuric acid, the reddish dichroic tint may be obtained, and the paper prepared with the solution as before.

The paper is used in the same way as ordinary litmus paper. A strip is moistened with distilled water and held as close as possible to the injection mass kept melted on a water-bath. It becomes blue at first, very rapidly and decidedly; but as fast as fresh quantities of acid are added this reaction becomes less evident, and at a certain moment the change of colour becomes very slow in making its appearance. It is then that the addition of acid should cease, and the operation is ended.

Very delicate sensitized paper may also be prepared with other reagents than litmus—for instance, with Nessler's reagent* or with alcoholic solution of hæmatoxylin, or with a

* Nessler's reagent may be prepared as follows:—Mercuric chloride, in powder, 35 grms.; iodide of potassium, 90 grms.; water, 1750 c.c. Heat gently till dissolved in a large basin; then add of stick caustic potash 320 grms., and 50 c.c. of saturated solution of mercuric chloride (WANKLYN). From COOLEY'S *Cyclopædia*, s. v. "Nessler's Test."

solution made by adding a trace of dilute sulphuric acid to "liqueur orange No. 3" (a liquid found in commerce, and used for detecting acids); the solution takes on a gooseberry-red colour.

The preparation of the injection mass is *facilitated* by employing acetic acid and ammonia of known strength. For the acetic acid it is sufficient to keep the glacial acid in a well-stoppered bottle. But this will not suffice for the ammonia, which is notably lowered in strength through the mere pouring from one bottle into another. Ville has imagined an apparatus which allows of withdrawing a known quantity without permitting any access of air to the stock solution. Description and figures, l. c.

With the exception of the processes above described, Ville prepares the injection mass exactly as Ranvier.

468. Gerlach's Carmine-Gelatin Mass (see *Arch. f. mik. Anat.*, 1865, p. 148; and Ranvier's *Traité*, p. 113).

469. Thiersch's Carmine-Gelatin Mass (see *ibid.*).

470. Carter's Carmine-Gelatin Mass (see BEALE, p. 113).

471. Davies' Carmine-Gelatin Mass (see his *Prep. and Mounting of Mic. Objects*, p. 138).

472. Hoyer's Carmine-Gelatin Mass (*Biol. Centralb.*, 1882, p. 21).—Take a concentrated gelatin solution and add to it a corresponding quantity of neutral carmine staining solution (see § 163). Digest in a water-bath until the dark violet-red colour begins to pass into a bright red tint. Then add 5—10 per cent. by volumes of glycerin, and at least 2 per cent. by weight of chloral, in a concentrated solution. After passing through flannel it can be kept in an open vessel under a bell-glass.

473. Fol's Carmine-Gelatin Mass (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 492).

The following method of preparation has the advantage of producing masses that can be kept in the *dry state* for an indefinite length of time. (Fol finds that the addition of chloral hydrate to wet masses is not an efficient preservative.)

One kilog. of Simeon's photographic gelatin* is soaked for

* This gelatin may be obtained either from the ordinary furnishers of

a couple of hours, until thoroughly soft, in a small quantity of water. The water is then poured off and the gelatin melted over a water-bath, and one litre of concentrated solution of carmine in ammonia is poured in with continual stirring. (The carmine solution is prepared by diluting strong solution of ammonia with three or four parts of water and adding carmine to saturation; the undissolved excess of carmine is removed by filtration just before the solution is added to the gelatin.)

To the mixture of gelatin and carmine, which should have a strong smell of ammonia, sufficient acetic acid is added to turn the dark purple colour of the mixture into the well-known blood-red hue. Exact neutralisation is not necessary. The mass is set aside until it has become firm, and is then cut up into pieces, which are tied up in a piece of tulle or fine netting. By means of energetic compression with the hand under water (it must be *acidulated* water, 0·1 per cent. acetic acid, otherwise the carmine will wash out: cf. *Journ. Roy. Mic. Soc.*, iv, part 3, 1884, p. 474) the mass is driven out through the meshes of the stuff in the shape of fine strings, which are washed for several hours in a sieve placed in running water in order to free them from any excess of acid or ammonia. The strings are then again melted, and the molten mass is poured on to large sheets of parchment paper soaked with paraffin, and the sheets are hung up to dry in an airy place. When dry the gelatin can easily be separated from the sheets, and may be cut into long strips with scissors and put away, protected from dust and damp, until wanted for use. In order to get the mass ready for use, all that is necessary is to soak the strips for a few minutes in water and melt them over a water-bath.

The process may be simplified, without giving very greatly inferior results, as follows (*Lehrb.*, p. 13). Gelatin in sheets is macerated for two days in the above-described carmine solution, then rinsed and put for a few hours into water acidulated with acetic acid. It is then washed on a sieve for several

articles used in photography, or direct from Simeon's Gelatin-fabrik, Winterthur, Switzerland. Two sorts, a hard and a soft, are sold; the softer is to be preferred on account of its lower point of fusion. Probably the photographic gelatins of Hinrichs, of Frankfurt, and of Coignet, of Paris, would answer equally well; as also the best English preparations.

hours in running water, dried on parchment paper, and preserved as above.

This injection mass is very well spoken of.

Blue Gelatin Masses.

474. Robin's Prussian Blue Gelatin Mass (see above, No. 462).

475. Ranvier's Prussian Blue Gelatin Mass (*Traité*, p. 119).—Twenty-five parts of a concentrated aqueous solution of soluble Prussian blue (prepared as directed below) mixed with 1 part of solid gelatin.

The mixture of the Prussian blue with the vehicle is effected in the following manner:

Weigh the gelatin, soak it in water for half an hour or an hour, wash it, and melt it in a test-tube, in the water it has absorbed, by heating over a water-bath. Put the solution of Prussian blue into another test-tube, and heat it on the same water-bath as the gelatin, so as to have the two at the same temperature. Pour the gelatin gradually into the Prussian blue solution, stirring continually with a glass rod. Continue stirring until the disappearance of the curdy precipitate that forms at first. (Some gelatins produce a *persistent* precipitate; these must be rejected; but it must be borne in mind that the precipitate that invariably forms in even the best gelatins disappears if the heating be continued. It is essential to remember this when preparing Prussian blue and gelatin mass.) As soon as the glass rod has ceased to show blue granulations on its surface on being withdrawn from the liquid, it may be concluded that the Prussian blue is completely dissolved. Filter through new flannel, and keep the filtrate at 40° over a water-bath until injected.

The soluble Prussian blue for the above mass is prepared as follows:

476. Soluble Prussian Blue for Injection Masses (Ranvier, *ibid.*).—Make a concentrated solution of sulphate of peroxide of iron in distilled water, and pour it gradually into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian blue. (An excess of prussiate of potash ought to remain in the liquid; in order to ascertain whether this is the case take a small

quantity of the liquid and observe whether a drop of sulphate of iron still precipitates it.) Filter the liquid through a felt strainer, underneath which is arranged a paper filter in a glass funnel. The liquid at first runs clear and yellowish into the lower funnel; distilled water is then poured little by little on to the strainer; gradually the liquid issuing from the strainer acquires a blue tinge, which, however, is not visible in that which issues from the lower filter. Distilled water is continually added to the strainer for some days until the liquid begins to run off blue from the second filter. The Prussian blue has now become soluble. The strainer is turned inside out and agitated in distilled water; the Prussian blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or the solution may be evaporated in a stove, and the solid residuum put away in bottles.

For injections, if a simple aqueous solution be taken, it should be *saturated*. Such a mass never transudes through the walls of vessels. Or it may be combined with one fourth of glycerin, or with the gelatin vehicle above described.

477. Soluble Prussian Blue (GUIGNET, *Journ. de Microgr.*, 1889, p. 94; *Journ. Roy. Mic. Soc.*, 1889, p. 468).—Guignet gives two methods:

1. To a boiling solution of 110 grammes of ferridcyanide of potassium are added gradually 70 grammes of crystallised sulphate of iron. After boiling two hours it is filtered, and the precipitate washed with fresh water until the washings are strongly blue. It is then dried at 100° C.

2. A saturated solution of oxalic acid is mixed to a pasty consistence with an excess of pure Prussian blue. The liquid is filtered and allowed to stand for two months until all the blue is precipitated. It is then filtered and washed with weak spirit in order to remove any oxalic acid, then dried.

A similar result may be at once obtained by precipitating the oxalic solution with 95 per cent. alcohol, or with a concentrated solution of sodium sulphate, and then washing the precipitate with weak spirit.

478. Brücke's Soluble Berlin Blue (*Arch. f. mik. Anat.*, 1865, p. 87).—Brücke first prepared it by taking a 10 per cent. solution of ferrocyanide of potassium, and precipitating by

means of a dilute solution of sesquichloride of iron (taken in such a quantity as to contain just half as much chlorine as is necessary for the decomposition), and washing the precipitate on the filter until solubility is attained.

Later on he employed a greater excess of ferrocyanide, and took just so much dilute solution of chloride of iron that the weight of the dry chloride employed came to $\frac{1}{10}$ or $\frac{1}{8}$ of that of the ferrocyanide. The precipitate was washed on a filter (using the filtrate to wash with) until nothing but a clear yellow liquid filtered off, then washed with water until the water began to run off blue, then dried, pressed between blotting-paper in a press, the resulting mass broken in pieces and dried by exposure to the air.

A cheaper method is the following:

Make a solution of ferrocyanide of potassium containing 217 grammes of the salt to 1 litre of water.

Make a solution of 1 part commercial chloride of iron in 10 parts water.

Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, and treat as above described.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold.

479. Thiersch's Prussian Blue Gelatin Mass (*Arch. f. mik. Anat.*, i, 1865, p. 148).

Take—

(1) A solution of 1 part gelatin in 2 parts water.

(2) A saturated aqueous solution of sulphate of iron.

(3) A saturated aqueous solution of red prussiate of potash.

(4) A saturated aqueous solution of oxalic acid.

Now (A) mix 12 c.c. of the iron solution with one ounce of the gelatin solution at the temperature of 25° R.

Then (B) mix, at the same temperature, 24 c.c. of the prussiate solution with two ounces of the gelatin solution.

(C) To the latter mixture add first 24 c.c. of the oxalic acid solution, stir well, and then add the gelatin and iron

mixture (A). Stir continually, keeping the temperature at from 20° to 25° R. until the whole of the Prussian blue is precipitated. Finally, heat over a water-bath to about 70° R. and filter through flannel.

480. Fol's Berlin Blue Gelatin Mass (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 494).—A modification of Thiersch's formula, No. 479. 120 c.c. of a cold saturated solution of sulphate of iron are mixed with 300 c.c. of the warm gelatin solution. In a separate vessel 600 c.c. of the gelatin solution are mixed with 240 c.c. of a saturated solution of oxalic acid, and 240 c.c. of a cold saturated solution of red prussiate of potash are added to the mixture. The first mixture is now gradually poured into the second, with vigorous shaking, the whole is warmed for a quarter of an hour over a boiling water-bath, the mass is allowed to set, is pressed out into strings through tulle or netting, as described for the carmine mass, *supra*, § 473, and the strings are washed and spread out to dry on the prepared paper. (It is necessary to dry the strings without remelting in this case, because the mass does not readily melt without the addition of oxalic acid.) In order to prepare the mass for injection, the strings are put to swell up in cold water, and then warmed with the addition of enough oxalic acid to allow of complete solution.

481. Hoyer's Soluble Berlin Blue Gelatin Mass (*Arch. f. mik. Anat.*, 1876, p. 649).—The filtered and not too much washed precipitate of soluble Berlin blue is brought in a little water on to a Graham's dialyser, and the external water changed until the solution begins to pass through the parchment. Dilute the solution and filter through filter-paper, an operation which becomes easy *after* dialysis. The solution may be injected pure (for lymphatics, for instance) or may be combined with gelatin. To do this, warm the solution almost to boiling-point, and add *gradually* a warm, thin solution of gelatin until coagulation begins to set in. Strain through wetted flannel.

Gelatin Masses of other Colours.

482. Robin's Cadmium Gelatin Mass (see § 463).

483. Thiersch's Lead Chromate Gelatin Mass (*Arch. f. mik. Anat.*, 1865, p. 149).

Make—

(A) A solution of 1 part gelatin in 2 parts water.

(B) A solution of 1 part neutral chromate of potash in 11 parts water.

(C) A solution of 1 part nitrate of lead in 11 parts water.

Mix 4 parts of the gelatin solution with 2 parts of the lead solution, and in another vessel mix 4 parts gelatin solution with 1 part of the chromate solution. Heat both the mixtures to 25° R.; mix them together with continual stirring until all the chromate of lead is precipitated; heat over a water-bath to 70° R., and filter through flannel.

484. Hoyer's Lead Chromate Gelatin Mass (*ibid.*, 1867, p. 136).

Take—

One volume of a solution of gelatin containing 1 part of gelatin to 4 of water.

One volume of cold saturated solution of bichromate of potash.

And one volume cold saturated solution of sugar of lead (neutral plumbic acetate).

Filter the gelatin solution through flannel, and mix in the bichromate solution. Then *warm almost to boiling-point*, and add gradually the (warmed) sugar of lead solution. Allow the mass to cool down to body temperature, and inject at once. Another mode of preparation is as follows:—Mix the sugar of lead solution with part of the gelatin solution, mix the bichromate solution with the remaining gelatin solution, heat the latter mixture, and pour into it the former mixture (gradually), stirring continually.

If the solutions are mixed at a low temperature a lumpy granular precipitate is formed. Further, when solution of sugar of lead is added to a *hot* solution of bichromate of potash a rich orange-red precipitate is obtained; whilst if the solutions be mixed *cold* the precipitate is bright yellow.

If the solutions of the two salts be kept ready prepared, the injection mass may be mixed in less than a quarter of an hour. Its advantages are that, on account of the extremely fine state of division of the precipitate, the mass is almost transparent, and runs so freely that even lymphatics may be perfectly injected with it, whilst its intensity of colour makes

the vessels much more distinct than the very pale mass of Thiersch (No. 483). It is also easier to manage than Thiersch's mass, as it does not solidify so quickly. It shows well in the vessels by reflected, as well as by transmitted light.

485. Fol's Lead Chromate Gelatin Mass (*Lehrb.*, p. 15).

486. Hoyer's Silver Nitrate Yellow Gelatin Mass (*Biol. Centralbl.*, ii, 1882, pp. 19, 22; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 142).—"A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid, which reduces the silver in a few seconds; chloral and glycerin are added as before" (see *ante*, HOYER'S formula for carmine-gelatin, No. 472).

This mass is yellow in the capillaries and brown in the larger vessels. It does not change either in alcohol, chromic or acetic acid, or bichromate of potash, &c.

487. Hoyer's Green Gelatin Masses (*ibid.*).—Made by mixing a blue mass and a yellow mass.

488. Thiersch's Green Gelatin Mass (*Arch. f. mik. Anat.*, 1865, p. 149).—Made by mixing the blue mass, § 479, and the yellow mass, § 483.

489. Robin's Scheele's Green Gelatin Mass (see § 464).

490. Hartig's White Gelatin Mass (FREY, *Le Microscope*, p. 190).—Dissolve 125 grammes of acetate of lead in so much water that the whole shall weigh 500 grammes.

Dissolve 95 grammes of carbonate of soda in so much water that the whole shall weigh 500 grammes.

Take equal volumes of the two solutions, and add two volumes of gelatin solution.

491. Frey's White Gelatin Mass (*ibid.*).—Put into a tall glass cylinder 125 to 185 grammes of cold saturated solution of chlorate of baryta. Add drop by drop, very carefully, sulphuric acid. Allow the precipitate that forms to settle for twelve hours, then decant almost all the clear supernatant liquid. The remaining mucilaginous mass containing the

precipitate is to be mixed with an equal part of concentrated gelatin solution.

Frey states that this is a very finely grained mass. Injected organs may be preserved in chromic acid.

492. Teichmann's White Gelatin Mass (*ibid.*, p. 191).—"Take 3 parts of nitrate of silver dissolved in the gelatin solution, and add 1 part of common salt."

The mass is very fine-grained, and is not decomposed by chromic acid; the disadvantage of it is that it blackens under the influnce of light and of sulphurous solutions.

493. Fol's Brown Gelatin Mass (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 494).—500 grms. of gelatin are soaked, and allowed to swell up, in 2 litres of water in which 140 grms. of common salt have previously been dissolved; the mass is melted over a water-bath, and a solution of 300 grms. of nitrate of silver in a litre of water is gradually added, with vigorous shaking. (If it be desired to have an extremely fine-grained mass, both the solutions should be diluted with 3 or 4 volumes of water.) The mass is pressed out into strings as before (§ 473), and the strings are stirred up in clear daylight with the following mixture:—1½ litres of cold saturated solution of potassic oxalate to 500 c.c. of cold saturated solution of sulphate of iron. As soon as the whole mass is thoroughly black the operation is at an end. The strings are then washed for several hours, remelted, and poured on to the prepared paper.

494. Miller's Purple Silver Nitrate Gelatin Mass.—See *Amer. Mon. Mic. Journ.*, 1888, p. 50; *Journ. Roy. Mic. Soc.*, 1888, p. 518; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 361.

495. Robin's Mahogany Gelatin Mass.—See § 461.

496. Ranvier's Gelatin Mass for Impregnation (*Traité*, p. 123).—Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

497. Fol's Metagelatin Vehicle (*Lehrb.*, p. 17).—The operation of injecting with the ordinary gelatin masses is greatly complicated by the necessity of injecting them warm. Fol proposes to employ metagelatin instead of gelatin.

If a slight proportion of ammonia be added to a solution of

gelatin, and the solution be heated for several hours, the solution passes into the state of metagelatin, that is, a state in which it no longer coagulates on cooling. Colouring masses may be added to this vehicle, which may also be thinned by the addition of weak alcohol. After injection, the preparations are thrown into strong alcohol or chromic acid, which sets the mass.

CHAPTER XXII.

INJECTIONS—OTHER MASSES.

498. Joseph's White-of-Egg Injection Mass (Carmine) (*Ber. naturw. sect. Schles. Ges.*, 1879, pp. 36—40; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 274).—"Filtered white of egg, diluted with 1 to 5 per cent. of carmine solution. . . . . This mass remains liquid when cold; it coagulates when immersed in dilute nitric acid, chromic or osmic acid, remains transparent, and is sufficiently indifferent to reagents."

For Invertebrates.

499. Bjeloussow's Gum Arabic Mass (*Arch. f. Anat. u. Phys.*, 1885, p. 379).—Make a syrupy solution of gum arabic and a saturated solution of borax in water. Mix the solutions in such proportions as to have in the mixture 1 part of borax to 2 of gum arabic. Rub up the transparent, almost insoluble mass with distilled water, added little by little, then force it through a fine-grained cloth. Repeat these operations until there is obtained a mass that is free from suspended gelatinous clots. (If the operation has been successful, the mass should coagulate in the presence of alcohol, undergoing at the same time a dilatation to twice its original volume.)

The vehicle thus prepared may be combined with any colouring mass except cadmium and cobalt.

After injection the preparation is thrown into alcohol, and the mass sets immediately, swelling up as above described, and consequently showing vessels largely distended.

Cold-blooded animals may be injected whilst alive with this mass. It does not flow out of cut vessels. Injections keep well in alcohol. Glycerin may be used for making them transparent.

If it be desired to remove the mass from any part of a preparation, this is easily done with dilute acetic acid, which dissolves it.

Glycerin Masses (cold).

500. Nitrite of Amyl as a Vaso-dilatator.—Glycerin masses are certainly very convenient, and give very good results from the scientific—not from the æsthetic—point of view. They have a great defect for the injection of fresh specimens—that is, those in which rigor mortis has not set in; they stimulate the contraction of arteries. In these cases it may be advisable to use nitrite of amyl as a vaso-dilatator. The animal may be anæsthetised with a mixture of ether and nitrite of amyl, and finally killed with pure nitrite. Or, after killing in any way, a little nitrite of amyl in salt solution may be injected before the injection mass is thrown in. In any case it is advisable to add a little nitrite to the mass just before using. The relaxing power is very great (see OVIATT and SARGENT, in *St. Louis Med. Journ.*, 1886, p. 207; and *Journ. Roy. Mic. Soc.*, 1887, p. 341).

501. Beale's Carmine Glycerin Mass (*How to Work, &c.*, p. 95).—Five grains of carmine are dissolved in a little water with the aid of about five drops of ammonia, and added to half an ounce of glycerin. Then add half an ounce of glycerin with eight or ten drops of acetic or hydrochloric acid, gradually, with agitation. Test with blue litmus paper, and if necessary add more acid till the reaction is decidedly acid. Then add half an ounce of glycerin, two drachms of alcohol, and six drachms of water.

502. Robin's Carmine Glycerin Mass (*Traité*, p. 33).—Consists of the following vehicle:

Glycerin	2 parts.
Alcohol	1 part.
Water	1 „

Combined with one third or one fourth its volume of the carmine colouring mass, *ante*, formula No. 460.

503. Beale's Prussian Blue Glycerin Mass (*How to Work, &c.*, p. 93).

Common glycerin . . .	1 ounce.
Spirits of wine	1 „
Ferrocyanide of potassium .	12 grains.
Tincture of perchloride of iron .	1 drachm.
Water	4 ounces.

Dissolve the ferrocyanide in one ounce of the water and glycerin, and add the tincture of iron to another ounce. "These solutions should be mixed together *very gradually* and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium.* Next, the spirit and the water are to be added very gradually, the mixture being constantly shaken."

"*The water*" spoken of in the last sentence appears to mean the remaining three ounces of water that were not mixed with the glycerin at first.

Injected specimens should be preserved in acidulated glycerin, otherwise the colour may fade.

504. Beale's Acid Prussian Blue Glycerin Mass (*ibid.*, p. 296).

Price's glycerine . . .	2 fluid ounces.
Tinct. of sesquichloride of iron .	10 drops.
Ferrocyanide of potassium . .	3 grains.
Strong hydrochloric acid . .	3 drops.
Water	1 ounce.

Proceed as directed above, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

I consider this a most admirable formula. I possess some of this mass prepared many years ago, in which not the smallest flocculus has made its appearance. The Prussian blue appears to be in a state of true solution. The mass runs well, and has not so much tendency to exude from cut capillaries as might be supposed.

505. Ranvier's Prussian Blue Glycerin Mass (*Traité*, p. 120).—Consists of the Prussian blue fluid, § 476, mixed with one fourth of glycerin.

506. Other Colours.—Any of the colouring masses, §§ 460 to 465, or other suitable colouring masses, combined with the vehicle, § 502.

Aqueous Masses.

507. Ranvier's Prussian Blue Aqueous Mass (*Traité*, p. 120).

—The soluble Prussian blue, § 476, injected without any vehicle. It does not extravasate.

508. Müller's Berlin Blue (*Arch. f. mik. Anat.*, 1865, p. 150). —Precipitate a concentrated solution of Berlin blue by means of 90 per cent. alcohol.

The precipitate is very finely divided; the fluid is *perfectly neutral*, and much easier to prepare than the formula of Beale.

509. Mayer's Berlin Blue (*Mitth. zool. Stat. Neapel*, 1888, p. 307).—A solution of 10 c.c. of tincture of perchloride of iron in 500 c.c. of water is added to a solution of 20 gr. of yellow prussiate of potash in 500 c.c. of water, allowed to stand for twelve hours, decanted, the deposit washed with distilled water on a filter until the washings come through dark blue (one to two days), and the blue dissolved in about a litre of water.

510. Emery's Aqueous Carmine (*ibid.*, 1881, p. 21).—To a 10 per cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red through incipient precipitation of the carmine. The supernatant clear solution is poured off, and injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of Fishes.

511. Letellier's Vanadate of Ammonia and Tannin (*Journ. Roy. Mic. Soc.*, 1889, p. 151).—Vanadate of ammonia is soluble in warm, and tannin in hot water. The two solutions are kept apart until required for use, when they are mixed according to the tint required. A black mass, very fine. The walls of vessels are stained black by it.

512. Taguchi's Indian Ink (*Arch. f. mik. Anat.*, 1888, p. 565; *Zeit. f. wiss. Mik.*, 1888, p. 503).—Chinese or (better) Japanese ink well rubbed up on a hone until a fluid is obtained that does not run when dropped on thin blotting-paper, nor form a grey ring round the drop. Inject until the preparation appears quite black, and throw it into some hardening liquid (not pure water).

I believe this will be found useful for many purposes, especially for work amongst Invertebrates, as well as for lymphatics, juice-canals, and the like.

Celloidin Masses.

513. Schiefferdecker's Celloidin Masses (*Arch. Anat. u. Phys.*, 1882 [*Anat. Abth.*], p. 201). (For Corrosion preparations.)

1. **Asphalt-celloidin** is the best of these injections. To prepare it—

Pulverise asphalt in a mortar, and put it for twenty-four hours into a well-closed vessel with some ether, shaking occasionally. After the twenty-four hours pour off the ether into another vessel, and dissolve in it small pieces of celloidin until the solution is of the consistency of one of the thicker fatty oils. (The undissolved asphalt may be employed for colouring a fresh quantity of ether, in which substance it is not very soluble.)

2. **Vesuvianin Celloidin Brown Injection.**—Make a concentrated solution of Vesuvianin in absolute alcohol, and dissolve celloidin in it. (This colour is not fast.)

3. **Opaque Blue Celloidin Injection.**—Dissolve celloidin in equal parts of absolute alcohol and ether, and add pulverised Berlin blue.

4. **Opaque Red Celloidin Injection.**—Proceed as above (3), taking pulverised cinnabar instead of Berlin blue. The last two pigments should be rubbed up in a mortar with a little absolute alcohol, and the paste added to the celloidin mass. Be careful not to take more pigment than is absolutely necessary, or the injection will become brittle. To filter (if this be thought necessary), strain the mass through flannel wetted with ether. Syringes must be free from grease, which would render the mass brittle. The nozzles to be filled with ether. Inject quickly, as the mass soon sets on contact with watery tissues. Clean syringes and nozzles with ether.

Corrosion of the Preparations.—The injected organs are thrown into unrectified hydrochloric acid, where they remain (the acid being changed from time to time if necessary) until

all the tissues are destroyed. Wash under a slow stream of water from a tap furnished with an india-rubber tube. Leave for some weeks in water, rinse, and put up in glycerin, or a mixture of glycerin, alcohol, and water in equal volumes.

514. Hochstetter's Modification of Schiefferdecker's Mass (*Anat. Anz.*, 1886, p. 51 ; *Journ. Roy. Mic. Soc.*, 1888, p. 159). —Kaolin is rubbed up with ether, to which cobalt blue, chrome yellow, or cinnabar is added. To this, celloidin solution of the consistence of honey is added.

Other Masses.

515. Budge's Asphaltum Mass (*Arch. f. mik. Anat.*, xiv, 1877, p. 70).—A large quantity of asphaltum has benzol poured on it, and is allowed to stand for several days, and then preserved for use. Before injecting add one third to one half benzol and filter. Chloroform and turpentine may also be used as solvents. Used for injecting the juice-canals of cartilage by the method described l. c., or by puncture.

516. Hoyer's Shellac Mass (*Arch. f. mik. Anat.*, 1876, p. 645). —Place a quantity of good shellac in a wide-necked flask, and add just enough alcohol (of about 80 per cent. strength) to cover the shellac. Leave it for twenty-four hours, and then warm it in a water-bath to complete the solution. When cool, dilute, if necessary, with alcohol to the consistency of a thin syrup, and strain through moderately thick muslin. The solution thus obtained may be coloured by the addition of anilin colours in (filtered) concentrated alcoholic solution, or of granular pigments suspended in alcohol. Of these, cinnabar gives the finest coloration, and may be employed for corrosion preparations (anilin colours may also be used for this purpose, but then they are not permanent). Berlin blue and yellow sulphide of arsenic are useful. A mixture of the two gives green. Freshly precipitated sulphide of cadmium gives a fine permanent yellow. The pigments should be rubbed up to fine powder with water, and alcohol added; let the mixture settle, pour off the dilute alcohol, and add strong alcohol. Shake in a flask, by which means the coarser particles are brought to the bottom of the liquid, and at this moment pour off the supernatant fluid which contains the finer particles

only. Add this to the shellac solution and strain through muslin. For very minute injections dilute the mixture with alcohol, filter through filter-paper on a covered funnel, and evaporate down to the desired consistency. Common moist water-colours, such as are sold in tin tubes, may be employed; they are to be well washed through several changes of water to get rid of the medium with which the pigments are mixed, and then suspended in alcohol as above directed. (These are to be recommended for injections into the blood of living animals.)

The shellac solution is not attacked by hydrochloric acid; hence its applicability to corrosion preparations. To correct the brittleness of the corroded mass it is well to add to the injection-fluid some 5 per cent. of a filtered alcoholic solution of Venetian turpentine. This may also be of use for preparations that are not to be corroded. For corrosion, concentrated (fuming) hydrochloric acid may be used, and small objects left in it for one day, large ones many days or even weeks.

For hardening injections, of which it is desired to cut sections, chromic acid may be used, or a mixture of chromic and hydrochloric acid (1 part of each to 250—500 parts water). Sections are best mounted in glycerin.

This method, with some slight modifications of detail, has lately been recommended by Bellarminow (*Anat. Anz.*, 1888, p. 650; see also *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 523, and *Journ. Roy. Mic. Soc.*, 1889, p. 150).

517. Hoyer's Oil-colour Masses (*Internat. Monatschr. f. Anat.*, 1887, p. 341; see also *Zeit. f. wiss. Mik.*, 1888, p. 80, and *Journ. Roy. Mic. Soc.*, 1888, p. 848).—5 grms. artist's Berlin blue oil-colour are rubbed up with 5 grms. thickened linseed oil, and mixed with about 30 grms. of lavender oil, fennel oil, thyme oil, or rosemary oil, allowed to stand for twenty-four hours in a well-stoppered vessel, and decanted. Shake before using.

For injection of the vessels of the spleen and other difficult objects.

Good results were also obtained with chrome-yellow oil-colour.

518. Pansch's Starch Mass (see *Arch. f. Anat. u. Entw.*, 1877, p. 480; 1880, pp. 232, 371; 1881, p. 76; 1882, p. 60; 1883, p. 265; and a modification of the same by GAGE, *Amer. Mon. Mic. Journ.*, 1888, p. 195; and *Journ. Roy. Mic. Soc.*, 1888, p. 1056).

519. Teichmann's Linseed-oil Masses (see *S. B. Math. Kl. Krakau Akad.*, vii, pp. 108, 158; *Journ. Roy. Mic. Soc.*, 1882, pp. 125 and 716).

520. Olive Oil for Corrosion Preparations (see below, § 558).

521. Natural Injections (ROBIN, *Traité*, p. 6).—To preserve these throw the organs into a liquid composed of 10 parts of tincture of perchloride of iron and 100 parts of water.

CHAPTER XXIII.

MACERATION AND DIGESTION.

Maceration.

522. Methods of Dissociation.—It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement-substances that may be present in the tissue. Simple teasing with needles is often insufficient to effect the desired isolation, as the cement-substances are often tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case recourse must be had to maceration processes, by which is here meant treatment with media which have the property of dissolving or at least softening the cement-substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia, for instance) admirable results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added, and the mount closed.

The student will do well not to neglect this simple method, which is one that it is most important to be acquainted with.

A good material for making *wax feet* is obtained (Vosseler,

Zeit. f. wiss. Mik., vii, 4, 1891, p. 461) by melting white wax and stirring into it one half to two thirds of Venice turpentine. Care must be taken if the operation be performed over a naked flame, as the turpentine vapours are inflammable.

523. Iodised Serum.—The preparation of this reagent has been given in Chap. XIX. The manner of employing it for maceration is as follows:—A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be completed by teasing or pressing out, as indicated above; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution, the maceration may be prolonged for several weeks.

It is obvious that these methods are intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent with regard to protoplasm, which it slightly hardens.

524. Artificial Iodised Serum (Frey, *Le Microscope*, p. 131; Ranvier, *Traité*, p. 77).

The formula has been given in Chap. XIX. Ranvier states that he has been unable to obtain good results, for purposes of maceration, by this method.

525. Alcohol.—Ranvier employs one-third alcohol (1 part of 36° alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. Ranvier finds that this mixture macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker ($\frac{1}{4}$ alcohol, for isolation of the nerve-fibres of the retina, for instance—*Thin*).

All observers are agreed that one-third alcohol is a macerating medium of the highest order; List (*Zeit. f. wiss. Mik.*, 1885, p. 511) states that for glandular structures it should be used with precaution, on account of swellings that it produces in the cells, and that Müller's solution, or osmic acid, should be preferred for such objects.

526. Salt Solution.—10 per cent. solution of sodium chloride is a well-known and valuable macerating medium.

527. Moleschott and Piso Borme's **Sodium Chloride and Alcohol** (Moleschott's *Untersuchungen zur Naturlehre*, xi, pp. 99—107; Ranvier, *Traité*, p. 242).—10 per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium, Ranvier finds the mixture inferior to one-third alcohol.

528. Chloral Hydrate.—In not too strong solution, from 2 to 5 per cent., for instance, chloral hydrate is a mild macerating agent that admirably preserves delicate elements. Lavdowsky (*Arch. f. mik. Anat.*, 1876, p. 359) recommends it greatly for salivary glands. Hickson (*Quart. Journ. Mic. Sci.*, 1885, p. 244) recommends it for the study of the retina of Arthropods.

529. Caustic Potash, Caustic Soda.—These solutions must be employed *strong*, 35 to 50 per cent. (Moleschott): so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however, be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. To make permanent preparations, the alkali should be neutralised by adding acetic acid, which forms with caustic potash acetate of potash, a well-known mounting medium (see Behrens, Kossel, and Schiefferdecker, *Das Mikroskop*, i, 1889, p. 156). It has been found by S. H. and S. P. Gage (*Proc. Amer. Soc. of Microscopists*, 1889, p. 35; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 349) that instead of acetic acid, 60 per cent. acetate of potash solution, employed in considerable quantity and if desired with addition of 1 per cent. of acetic acid, may be used, the preparations either being mounted therein, or in glycerin or glycerin jelly. They may be stained if the acetate be first washed out by treatment for twenty-four hours with alum solution.

530. Sulphocyanides of Ammonium and Potassium (Stirling, *Journ. Anat. and Phys.*, xvii, 1883, p. 208).—Ten per cent. solution of either of these salts is an admirable dissociating medium for epithelium. Macerate small pieces for twenty-

four to forty-eight hours, stain with fuchsin, eosin, or picro-carmine.

If a crystalline lens be macerated as above its fibres become beaded or moniliform.

531. Saliva, Artificial (for embryology of nerve and muscle) (CALBERLA'S formulæ, *Arch. f. mik. Anat.*, xvi, 1879, p. 471, *et seq.*).—After having made trial of various different macerating agents, with the object of obtaining isolation of the developing muscle and nerve of embryos of *Amphibia* and *Ophidia*, Calberla found that the best results were obtained by means of Czerny's mixture of saliva and solutio Mülleri. This led him to imagine an artificial saliva, which on trial gave results as good as those obtained by natural saliva, or even better.

Second formula (the first formula is suppressed, as being more complicated, and not giving better results):

Potassium chloride	0·4
Sodium chloride	0·3
Phosphate of soda	0·2
Calcium chloride	0·2
	1·1

This is dissolved in 100 parts of water, saturated with carbonic acid, and the solution combined with water and solutio Mülleri, one volume of the solution being combined with half a volume of Müller's solution and a volume of water.

In either case the Müller's solution may be replaced by a 2½ per cent. solution of chromate of ammonia. The best results were obtained when the solutions were saturated with the CO_2 just before using.

The tissues are isolated by teasing and shaking, and specimens mounted in concentrated acetate of potash.

532. LANDOIS'S **Solution** (*Arch. f. mik. Anat.*, 1885, p. 445).

Saturated sol. of neutral chromate of ammonia	5 parts.
Saturated sol. of phosphate of potash . .	5 „
Saturated sol. of sulphate of soda . . .	5 „
Distilled water	100 „

To be used in the same way as chromic acid:—Small pieces of tissue are macerated for one to three, or even four or five

days, in the liquid, then brought for twenty-four hours into ammonia carmine diluted with one volume of the macerating liquid.

Gierke particularly recommends this liquid for all sorts of macerations, but especially for the central nervous system, for which he finds it superior to all other agents. It is also recommended for the same purpose by Nansen (v. *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 242).

533. Permanganate of Potash.—Has an action similar to that of osmic acid, but more energetic. Is recommended, either alone or combined with alum, as the best dissociating agent for the fibres of the cornea (Rollett, *Stricker's Handbuch*, p. 1108).

534. Chromic Acid.—Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve-tissue. About 10 c.c. of the solution should be taken for a cube of 5 mm. of the tissue (Ranvier).

535. Bichromate of Potash.—0·2 per cent.

536. Müller's Solution.—Same strength.

537. Müller's Solution and Saliva (see above, § 531).

538. Brock's **Medium** (for nervous system of Mollusca, *Intern. Monatsch. f. Anat.*, i, 1884, p. 349).—Equal parts of 10 per cent. solution of bichromate of potash and visceral fluid of the animal.

539. Möbius's **Media** (quoted from *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 402).

1. One part of sea water with 4 to 6 parts of 0·5 per cent. solution of bichromate of potash.

2. 0·25 per cent. chromic acid, 0·1 per cent. osmic acid, 0·1 per cent. acetic acid, dissolved in sea water. For Lamellibranchiata. Macerate for several days.

540. Gage's **Picric Alcohol** (*Proc. Amer. Soc. of Microscopists*, 1890, p. 120; *Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 87, 88).—95 per cent. alcohol, 250 parts; water, 750; picric acid, 1. Recommended for most tissues, but especially for epithelia and smooth and striated muscle. This is also much recom-

mended by HOPKINS in the same place. A few hours' maceration is generally sufficient.

541. Osmic Acid.—0·1 per cent., for from a few minutes to a fortnight (cortex of cerebrum—Rindfleisch). May be followed by maceration in glycerin.

542. Osmic and Acetic Acid (the HERTWIGS' *Liquid, Das Nervensystem u. die Sinnesorgane der Medusen*, Leipzig, 1878, and *Jen. Zeitschr.*, xiii, 1879, p. 457; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 441, and [N.S.] iii, 1883, p. 732).

0·05 per cent.	osmic acid	. . .	1 part.
0·2 „	acetic acid	. . .	1 „

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed; they then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in Beale's carmine (in order to prevent the osmium from over-blackening, and to assist the maceration), and are preserved in glycerin.

For *Actiniæ* the osmic acid is taken weaker, 0·04 per cent.; both the solutions are made with sea water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with Beale's carmine.

543. BÉLA HALLER'S **Mixture** (*Morphol. Jahrb.*, xi, p. 321).—One part glacial acetic acid, 1 part glycerin, 2 parts water. Specially recommended for the central nervous system of Mollusca (Rhipidoglossa). A sufficient degree of maceration is obtained in thirty to forty minutes, the cells showing less shrinkage than with other liquids.

544. Nitric Acid.—Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube. Preparations may afterwards be washed with water and put up in strong solution of alum, in which they may be preserved for a long time (HOPKINS, *Proc. Amer. Soc. of Microscopists*, 1890, p. 165; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 86).

Maceration is greatly aided by heat, and at a temperature

of 40° to 50° C. may be sufficiently complete in an hour (GAGE).

545. Nitric Acid and Chlorate of Potash (KÜHNE's method, *Ueber die peripherischen Endorgane*, &c., 1862; RANVIER, *Traité*, p. 79).—Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

546. Sulphuric Acid (RANVIER, *Traité*, p. 78).—Sulphuric acid has been employed by Max Schultze for isolating the fibres of the crystalline.

Macerate for twenty-four hours in 30 grms. of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate.

ODENIUS found very dilute sulphuric acid to be the best reagent for the study of nerve-endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grains of "English sulphuric acid" to the ounce of water.

Hot concentrated sulphuric acid serves to dissociate horny epidermic structures (horn, hair, nails).

547. Oxalic Acid.—Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

548. SCHIEFFERDECKER'S **Methyl Mixture** (for the retina) (*Arch. f. mik. Anat.*, xxviii, 1886, p. 305).—Ten parts of glycerin, 1 part of methyl alcohol, and 20 parts of distilled water. Macerate for several days (perfectly fresh tissue).

Digestion.

549. BEALE'S **Digestion Fluid** (*Archives of Medicine*, i, 1858, pp. 296—316).—The mucus expressed from the stomach glands of the pig is rapidly dried on glass plates, powdered, and kept in stoppered bottles. It retains its properties for years. Eight tenths of a grain will dissolve 100 grains of coagulated white of egg.

To prepare the digestion fluid, the powder is dissolved in

distilled water, and the solution filtered. It filters readily. Or the powder may be dissolved in glycerin. The tissues to be digested may be kept for some hours in the liquid at a temperature of 100° F. (37° C.).

550. BRÜCKE'S **Digestion Fluid** (from CARNOY'S *Biologie cellulaire*, p. 94).

Glycerinated extract of pig's stomach.	1 vol.
0·2 per cent. solution of HCl	3 vols.
Thymol, a few crystals.	

551. BICKFALVI'S **Digestion Fluid** (*Centralbl. f. d. med. Wiss.*, 1883, p. 833).—One grm. of dried stomachal mucosa is mixed with 20 c.c. of 0·5 per cent. hydrochloric acid, and put into an incubator for three or four hours, then filtered. Macerate the tissue in the solution for not more than half an hour to an hour.

552. KUSKOW'S **Digestion Fluid** (*Arch. f. mik. Anat.*, xxx. p. 32; cf. *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 384).—One part of pepsin dissolved in 200 parts of 3 per cent. solution of oxalic acid. The solution should be freshly prepared, and the objects (sections of hardened Ligamentum Nuchæ) remain in it at the ordinary temperature for ten to forty minutes.

553. SCHIEFFERDECKER'S **Pancreatin Digestion Fluid** (*Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 483).—Solution of pancreatin in water. Schiefferdecker employs the "Pankreatinum siccum" prepared by Dr. Witte, Rostock. A saturated solution is made in distilled water, cold, and filtered. Pieces of tissue (epidermis) are macerated in it for three to four hours at about body temperature. Nuclei are preserved, and the forms of prickle-cells well shown.

554. KÜHNE'S **Methods** (see *Unters. a. d. Phys. Inst. Univ. Heidelberg*, i, 2, 1877, p. 219).—Trypsin is prepared by extracting ox-pancreas with alcohol and ether, and evaporating to dryness. One part of the product is heated for three to four hours at a temperature of 40° C., with 5 to 10 parts of 0·1 per cent. solution of salicylic acid, and the solution forced through linen and filtered cold.

554a. GEDOELST'S **Methods** (see *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126; also *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 57).

CHAPTER XXIV.

CORROSION, DECALCIFICATION, AND BLEACHING.

Corrosion.

555. Caustic Potash, Caustic Soda, Nitric Acid.—Boiling, or long soaking in a strong solution of either of these, is an efficient means of removing soft parts from skeletal structures (appendages of Arthropods, spicula of sponges, &c.).

556. Eau de Javelle (Hypochlorite of Potash) (NOLL'S METHOD, *Zool. Anzeig.*, 122, 1882, p. 528).—Noll remarks that the usual method of preparing the skeleton of siliceous sponges and similar structures by corroding away the soft parts by means of caustic potash has many disadvantages, of which a principal one is that the spicula are not preserved in their normal positions. He therefore proceeds as follows:—A piece of sponge is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, and treated with successive alcohols and oil of cloves, and finally mounted in balsam.

The same process is stated to be applicable to calcareous structures. I feel convinced, however, that if the structures are *delicate*, they will suffer, or be entirely destroyed.

557. Eau de Labarraque (Hypochlorite of Soda) may be used in the same way as eau de Javelle. LOOSS (*Zool. Anz.*, 1885, p. 333) finds that either of these solutions will completely dissolve chitin in a short time with the aid of heat. For this purpose the commercial solution should be taken concentrated and boiling. A formula for making it is given in § 581.

If solutions diluted with 4 to 6 volumes of water be taken, and chitinous structures be macerated in them for twenty-

four hours or more, according to size, the chitin is not dissolved, but becomes transparent, soft, and permeable to staining fluids, aqueous as well as alcoholic. The most delicate structures, such as nerve-endings, are stated not to be injured by the treatment. The method is applicable to Nematodes and their ova, an object well known for the resistance they oppose to ordinary reagents.

This is undoubtedly a valuable method.

558. Altmann's **Corrosion Method** (*Arch. f. mik. Anat.*, 1879, p. 471). —Whilst almost all animal tissues are very quickly destroyed by eau de Javelle, yet fats, and particularly fats hardened by osmic acid, withstand its action for a long time. If, then, you introduce some fat or other into a tissue, harden it with osmic acid and corrode the tissue with eau de Javelle, you will obtain a mould in osmium-blackened and hardened fat, of the spaces you had filled with the fat introduced.

The method may be of much use in certain special researches, such as those on the choroid, iris, and pigmented organs. I recommend the reader to carefully study the article, which does not well bear abstracting. A good abstract will be found in *Journ. Roy. Mic. Soc.*, 1879, p. 610, with plate.

Decalcification and Desilicification.

559. Decalcification of Bone.—I take the following historical sketch from Busch's article "On the Technique of the Histology of Bone" (*Arch. f. mik. Anat.*, xiv, 1877, p. 481; see also the paper of Haug, in *Zeit. f. wiss. Mik.*, viii, i, 1891, p. 1).

The most widely used agent for decalcification is *hydrochloric* acid. Its action is rapid, even when very dilute, but it has the disadvantage of causing serious swelling of the tissues. To remedy this chromic acid may be combined with it, or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per cent. solution of *chloride of palladium* may be added $\frac{1}{10}$ of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. Fo this latter reason it can never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic, and *pyroligneous acids* have considerable

decalcifying power, but cause great swelling. *Picric acid* has a very slow action, and is only suitable for very small structures.

560. Nitric Acid (Busch, l. c.).—To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously, whilst at the same time it does not injuriously attack tissue-elements.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 vols. water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily,* for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or chromate of potash (1 per cent.). By putting them afterwards into alcohol the well-known green stain is obtained.

Staining agents.—Sections of bone treated in the last-described manner are stained five or ten minutes in a weak aqueous solution of eosin. The ground-substance and small cells of cartilage remain colourless, the nuclei of the large cells are stained red, and so is periosteum, bone-tissue, and the cellular contents of the medullary spaces. Hæmatoxylin may be used in conjunction with eosin (before or after it) to obtain double-stains, which, however, are seldom successful.

Sections are dehydrated in absolute alcohol, and mounted (*without* clearing by oil of cloves or the like) in a benzol-solution of Canada balsam.

561. Nitric Acid and Alcohol.—3 per cent. of nitric acid in 70 per cent. alcohol. Soak specimens for several days or weeks. I do not know who first recommended this admirable medium.

THOMA (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 191) gives the following method.—Take 5 vols. of 95 per cent. alcohol and 1 vol. pure concentrated nitric acid. Leave bones in this mixture, changing the liquid every two or three days, until thoroughly decalcified, which should happen even with large bones in two or three weeks. Wash out until every trace of acid is removed (*i. e.* for some days after no acid reaction is obtained with litmus paper) in 95 per cent. alcohol containing an excess of precipitated chalk. This may take eight to fourteen days, after which the tissues will stain well and may be treated as desired.

562. Phloroglucin (ANDEER, *Centralbl. f. d. med. Wiss.*, xii, xxxiii, pp. 193, 579; *Intern. Monatschr.*, i, p. 350; *Zeit. f. wiss. Mik.*, 1885, pp. 375, 539; *Journ. Roy. Mic. Soc.*, 1887, p. 504; HAUG, *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 8; FERRERI, *ibid.*, ix, 2, 1892, p. 236; *Bull. R. Accad. Med. di Roma*, 1892, p. 67).—This is the most recent of the decalcification methods. It has the advantages of being the most rapid of any, and of preserving the tissues very well (with the exception of blood).

Phloroglucin by itself is not a solvent of lime salts; its function in the mixtures given below is so to protect the organic elements of tissues against the action of the mineral acids that these can be used in a much more concentrated form than would otherwise be advisable.

HAUG advises the following procedure:—Bring 1 grm. of phloroglucin into 10 c.c. of pure, not fuming nitric acid (1·4 sp. gr.), and warm very slowly and carefully with gentle agitation. There is formed a clear solution of (presumably) a nitrate of phloroglucin. Dilute the solution with 100 c.c. of distilled water, and add 10 c.c. of nitric acid. This gives a solution containing 20 per cent. of acid, which is the proper proportion. More water may be added to the solution, to make it up to 300 c.c., if nitric acid be also added in the proportion given. But the dilution must not be carried beyond this point, in order that the preservative action of the phloroglucin be not overmuch weakened. The process of decalcification in this solution is extremely rapid, and therefore should be carefully watched. Fœtal and young bones become quite soft in half an hour; small pieces of old and hard bones (femur, temporal bone) in a few hours. Teeth

take longer, and may require, if time be an object, a solution made with 35 to 45 per cent. of nitric acid. Wash out for two days in running water. The tissues stain well.

The solution may be made with hydrochloric acid instead of nitric acid, 30 per cent. of acid being taken, and 0·5 per cent. of sodium chloride added.

For slow decalcification a 2 to 5 per cent. nitric acid solution may be used, or a mixture containing of phloroglucin 1 part, nitric acid 5, alcohol 70, and distilled water, 30 parts.

For the labyrinth, FERRERI advises a mixture containing 1 grm. of phloroglucin, 10 grms. of hydrochloric acid, 100 of water, and 200 of 70 per cent. alcohol, to be changed once a week during thirty to forty days.

563. Hydrochloric Acid (see above, § 559).—RANVIER says that it may be taken of 50 per cent. strength, and then has a very rapid action. To counteract the swelling action of the acid, sodium chloride may be added. Two formulæ of this sort have been given by VON EBNER (see HAUG's paper quoted in the last section). The first is, 100 c.c. cold saturated solution of sodium chloride in water, 100 c.c. water, and 4 c.c. hydrochloric acid. Preparations to be placed in this, and 1 to 2 c.c. of hydrochloric acid added daily until they are soft. The second is, 2·5 parts of hydrochloric acid, 500 of alcohol, 100 of water, and 2·5 of sodium chloride. HAUG prefers the proportions of 1·0 to 5·0 of acid, 70 of alcohol, 30 of water, and 0·5 of salt.

564. Hydrochloric Acid and Chromic Acid (BAYERL, *Arch. f. mik. Anat.*, 1885, p. 35).—Equal parts of 3 per cent. chromic acid and 1 per cent. hydrochloric acid. For ossifying cartilage. HAUG recommends equal parts of 1 per cent. hydrochloric acid and 1 per cent. chromic acid (l. c.).

565. Hydrochloric Acid and Nitric Acid (HOPEWELL SMITH, *Journ. Roy. Mic. Soc.*, 1892, p. 433).—Place teeth in 12 parts of 10 per cent. HCl, and after 15 hours add 1·5 parts of HNO_3, and after 48 hours add 1·5 parts more of HNO_3. After 75 to 80 hours remove and wash for half an hour in a solution of 5 grms. of lithium carbonate to an ounce of water.

566. Hydrochloric Acid and Glycerin.—Glycerin, 95; hydrochloric acid, 5. Recommended for softening teeth in SQUIRE's *Methods and Formulæ*, p. 12.

567. Picric Acid should be taken saturated.

Picro-sulphuric acid should of course be avoided on account of the formation of gypsum.

Picro-nitric or Picro-hydrochloric Acid.—The reader will perhaps reflect that the last two fluids appear likely to be very useful for decalcifications. Mayer points out that the action is very rapid, and that the copiously evolved CO_2 often produces, mechanically, lesions in tissues; so that in many cases in which calcareous structures are concerned chromic acid is to be preferred,

the more so as it more effectually hinders any *collapsing* of the structures that might result from the withdrawal of their supporting calcareous elements.

Picric acid fluids are good media, as though their action is slow they preserve tissues well, and leave them in a good state for subsequent staining.

568. Phosphoric Acid.—10 to 15 per cent. (HAUG, l. c., in § 559). Somewhat slow, staining not good.

569. Lactic Acid.—10 per cent. or more. Fairly rapid, preserves well, and may be recommended (HAUG, l. c.).

570. Chromic Acid is employed in strengths of from 0·1 per cent. to 2 per cent., the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually. In any way the action is extremely slow, and it is therefore better to take one of the mixtures of chromic acid with a more energetic agent.

571. Chromic and Nitric Acid.—Dissolve 15 grms. pure chromic acid in 7 oz. of distilled water, to which 30 minims of nitric acid are afterwards to be added. Macerate for three or four weeks, changing the fluid frequently (Marsh).

FOL takes 70 volumes of 1 per cent. chromic acid, 3 of nitric acid, and 200 of water (*Lehrb.*, p. 112).

See also § 564. It remains to be added that even with the addition of nitric or hydrochloric acid the action is excessively slow, frequently requiring months to be complete.

572. Arsenic Acid.—4 per cent. aqueous solution, used at a temperature of 30° to 40° C. (SQUIRE's *Methods and Formulæ*, &c., p. 11).

573. Glycerin. Alum-Carmine.—It should be remembered that these commonly used reagents dissolve carbonate of lime; they must therefore be avoided in the preparation of structures containing delicate calcareous elements that it is wished to preserve (calcareous sponges, larvæ of Echinodermata, &c.).

Desilicification.

574. Hydrofluoric Acid (MAYER's method, *Zool. Anz.*, 1881, No. 97, p. 593).—The objects from which it is desired to remove siliceous parts are brought in alcohol into a glass vessel coated internally with paraffin (otherwise the glass would be corroded by the acid). Hydrofluoric acid is then added drop by drop (the operator taking great care to avoid the fumes, which attack mucous membranes with great energy). A *Wagnerella borealis* may thus be completely desilicified in a few minutes. Small pieces of siliceous sponges will require a few hours, or at most a day. The tissues do not suffer; and if they have been previously stained with acetic acid carmine the stain does not suffer; at least, this was so in the case of *Wagnerella*.

This dangerous method is best avoided. As regards sponges, I would point out that if well imbedded, good sections may be made from them with-

out previous removal of the spicula. The spicula appear to be cut; probably they break very sharply when touched by the knife. Knives are of course not improved by cutting such sections.

Bleaching.

575. MAYER'S **Chlorine Method** (*Mitth. Zool. Stat. Neapel,* ii, 1881, p. 8).—This is a process imagined for the purpose of getting rid of the blackening that often occurs as a consequence of treatment by osmic acid.

The specimens are put into alcohol (either of 70 or 90 per cent.). Crystals of chlorate of potash are added until the bottom of the vessel is covered with them. A few drops of concentrated hydrochloric acid are then added by means of a pipette, and mixed in by shaking the vessel as soon as the green colour of the evolving chlorine has begun to show itself. Warm if necessary; but most objects, even large ones, may be bleached in half a day without the employment of heat. The tissues do not suffer.

Instead of hydrochloric acid, nitric acid may be used; in which case the bleaching agent is the freed oxygen, instead of chlorine.

The first method may be used for the purpose of removing pigment from the eyes of insects.

576. MARSH'S **Chlorine Method** (*Section Cutting,* p. 89).—Marsh generates chlorine in a small bottle by treating crystals of chlorate of potash with strong HCl, and leads the gas (by means of a piece of glass tubing bent twice at right angles) to the bottom of a bottle containing the sections in water. (See a fig. of the apparatus in *Journ. Roy. Mic. Soc.,* iii, 1880, p. 854.)

577. Chlorine Solution (SARGENT'S method).—Hydrochloric acid, 10 drops; chlorate of potash, ½ dr.; water, 1 oz. Soak for a day or two. Wash well.

This method is intended for "bleaching insects;" it will be seen that it is only applicable to the preparation of hard parts, as soft tissues would be destroyed by the solution.

578. Kreasote (POUCHET'S method, *Journ. de l'Anat.,* 1876, p. 8, *et seq.*).—I gather from the paper here quoted that most of the granular animal pigments are soluble in kreasote. Other solvents are mentioned in this paper ("On the Change of Coloration through Nervous Influence"), but this appears to be the only one capable of general histological application.

579. Nitric Acid has a similar action. PARKER (*Bull. Mus. Comp. Zool.*, Cambridge, U.S.A., 1889, p. 173; see *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 82) says that for eyes of scorpions the usual 5 to 10 per cent. solutions are not strong enough. He treats sections, fixed to the slide with Schällibaum's medium, for about a minute with a solution of up to 50 per cent. of nitric acid in alcohol, or, still better, with a 35 per cent. solution of a mixture of equal parts of nitric and hydrochloric acid in alcohol. To make the solution, the acid should be poured slowly into the alcohol (not *vice versâ*), and the mixture kept cool.

580. Peroxide of Hydrogen (Oxygenated Water) (POUCHET'S method, M. DUVAL, *Précis*, &c., p. 234).—Macerate in glycerin to which has been added a little oxygenated water (5 to 6 drops to a watch-glass of glycerin). (Oxygenated water may be procured from perfumers or hair-dressers, by whom it is sold as a hair dye under the name of "Auréoline," "Golden hair-wash," or the like.)

The brownish-green colour communicated to tissues by chromic solutions may be changed to yellow by means of oxygenated water (see § 30).

Osmium preparations may be bleached in the same way.

581. Eau de Labarraque. Eau de Javelle (see §§ 556, 557).—These are bleaching agents. For the manner of preparing a similar solution see *Journ. de Microgr.*, 1887, p. 154, or *Journ. Roy. Mic. Soc.*, 1887, p. 518. It is, shortly, as follows:—8 parts of caustic soda are dissolved in 100 parts of distilled water, and chlorine is passed through to saturation. During the passage of the chlorine the solution must be surrounded with a mixture of salt and ice, otherwise the temperature rises, and chloride and chlorate of soda are produced. The resulting solution contains 7·45 per cent. of hypochlorite of soda. It is green; and the more effectual the cold, the greener is the colour. The energy of the decolourising action is proportional to the greenness of the solution. Of course the method cannot be used for bleaching soft parts which it is desired to preserve.

582. Chloroform helps to clear strongly pigmented chitin, and combined with nitric acid will decolourise it entirely (see below, in the chapter on Arthropods, Part II).

583. GRENACHER'S **Mixture for Eyes of Arthropods and other Animals** (*Abh. nat. Ges. Halle-a.-S.*, xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244).

Glycerin . . . . . 1 part.
80 per cent. alcohol . . . . 2 parts.
Mix and add 2 to 3 per cent. of hydrochloric acid.

Pigments dissolve in this fluid, and so doing form a stain which suffices in twelve to twenty-four hours for staining the nuclei of the preparation. You may, if you like, first stain the objects with borax-carmine, and then put them into the liquid, the pigment being washed out more rapidly than the carmine. But the progress of the decoloration must be carefully watched.

PART II.

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXV.

EMBRYOLOGICAL METHODS.

584. Artificial Fecundation.—This practice, which affords the readiest means of obtaining the early stages of development of many animals, may be very easily carried out in the case of the Amphibia anura, Teleostea, Cyclostomata, Echinodermata, and many Vermes and Cœlenterata.

In the case of the Amphibia, both the female and the male should be laid open, and the ova should be extracted from the uterus and placed in a watch-glass or dissecting-dish, and treated with water in which the testes, or, better, the vasa deferentia, of the male have been teased.

Females of Teleostea are easily spawned by manipulating the belly with a gentle pressure; and the milt may be obtained from the males in the same way. (It may occasionally be necessary, as in the case of the Stickleback, to kill the male, and dissect out the testes and tease them.) The spermatozoa of fish, especially those of the Salmonidæ, lose their vitality very rapidly in water; it is therefore advisable to add the milt immediately to the spawned ova, then add a little water, and after a few minutes put the whole into a suitable hatching apparatus with running water.

Artificial fecundation of Invertebrates is easily performed in a similar way. It is sometimes possible to perform the operation under the microscope, and so observe the penetration of the spermatozoon and some of the subsequent phenomena, as has been done by Fol, the Hertwigs, Selenka, and others, for the Echinodermata and other forms.

585. Superficial Examination.—The development of some animals, particularly some Invertebrates, may be to a certain extent followed by observation of the living ova under the microscope. This may usefully be done in the case of various Teleosteans, such as the Stickleback, the Perch, *Macropodus*,

and several pelagic forms, and with *Chironomus*, *Asellus aquaticus*, Ascidians, *Planorbis*, many Cœlenterata, &c. I advise the student to carefully draw the different stages so observed, for such drawings are most important aids to the study of the same stages by the section method.

Some ova of Insecta and Arachnida which are completely opaque under normal conditions become transparent if they are placed in a drop of oil; if care be taken to let their surface be simply impregnated with the oil, the normal course of development is not interfered with (Balbiani).

586. Preparation of Sections.—Osmic acid, employed either alone or in combination with other reagents, is an excellent fixing agent for small embryos, but not at all a good one for large ones. It causes cellular elements to shrink somewhat, and therefore brings out very clearly the slits that separate germinal layers, and any channels or other cavities that may be in course of formation.

In virtue of its property of blackening fatty matters, myelin amongst them, it is of service in the study of the development of the nervous system.

Chromic acid is indispensable for the study of the external forms of embryos; it brings out elevations and depressions clearly, and preserves admirably the mutual relations of the parts; but it does not always preserve the forms of cells faithfully, and is a hindrance to staining in the mass.

Picric liquids have an action which is the opposite of that of osmic acid; they cause cellular elements to swell somewhat, and thus have a tendency to obliterate spaces that may exist in the tissues. But notwithstanding this defect, the picric compounds, and especially Kleinenberg's picro-sulphuric acid, are amongst the best of embryological fixing agents.

For imbedding, the celloidin-chloroform method of Viallanes gives excellent results, and so does paraffin. This latter is preferable in so far as it lends itself better to the rapid production of series of sections, and allows of the use of the Cambridge Rocking Microtome, or the Minot, which is perhaps the microtome *par excellence* of the embryologist.

As to staining, my eminent fellow-worker, Dr. Henneguy, writing the chapter on embryological methods for the French

edition of this work, advised staining in the mass with borax-carmine or alum-carmine (Henneguy's acetic acid formula, § 156); or, as an alternative, the staining of sections by Flemming's method. The improvements that have in recent times been worked out in this method give still greater weight to the latter recommendation.

587. Reconstruction of Embryos from Sections.—The study of a series of sections of any highly differentiated organism of unknown structure is so complicated that it is often necessary to have recourse to elaborate methods of geometrical or of plastic reconstruction in order to obtain an idea or a model of the whole. These methods have now been brought to so high a degree of complexity that a volume rather than a paragraph would be necessary to describe them. See Born, "Die Plattenmodellirmethode," in *Arch. f. mik. Anat.*, 1883, p. 591, and *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 433; Strasser, in *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 179, and iv, 2 and 3, pp. 168 and 330; Kastschenko, in *Zeit. f. wiss. Mik.*, iv, 2 and 3, 1887, pp. 235–6 and 353, and v, 2, 1888, p. 173 (abstracts of all these papers may be found in *Journ. Roy. Mic. Soc.* of the years quoted).

A simple, but in many cases quite efficient plan, has been described by Fol (*Lehrb.*, p. 35) as follows:—Before cutting your sections, you make an outline drawing of your object, under the magnification that you intend to employ for the reconstructed drawing, and in a plane perpendicular to that of the intended sections. For instance, if you intend to make transverse sections of an embryo, begin by making a profile drawing of it, that is, a drawing of the outline of an ideal sagittal section of it. Then make your series of sections, and make drawings of them all under the same magnification as the sagittal drawing. Then trace over your sagittal drawing a series of equidistant parallel lines in positions corresponding to the sections that have been made. If your sections are one hundredth of a millimetre thick, and your drawing be magnified one hundred times, the lines should be one millimetre apart (if you intend to reconstruct the whole of your sections, but the operation may frequently be abridged by only reconstructing say every fifth or every tenth section).

You have now to fill in your outline drawing with details borrowed from the drawings of the sections. You may help yourself greatly in the following way:—A plate of glass, of a size suitable to the intended drawings, is covered with a layer of gelatin, and dried. On this is ruled a series of parallel lines, very close together, and ruled with differently coloured inks, the colours recurring in regular order. The plate is then cut into two unequal parts by a diamond, on a line perpendicular to the coloured lines.

Lay one of the parts of the plate on the outline drawing so that the cut edge covers the line that corresponds to the first section you are going to fill in ; then lay the other part of the plate on the drawing of the section in such a position that the limits of the drawing correspond to the same coloured lines that cover the limits of the outline drawing on the other part of the plate already placed.

Trace on the plate that covers the drawing of the section the outline of the internal organs. Lay it against its fellow-plate on the outline drawing, making the coloured lines correspond, and you will easily be able to mark off accurately on the outline drawing a series of dots that correspond in position to the outlines of the internal organs. This operation having been repeated for each of the sections that you desire to bring into your reconstruction, nothing remains but to join your dots by lines, and you will have filled up your outline drawing with a representation of the internal organs in the same plane.

If any reader think this process complicated, he needs but to spend five minutes in trying it with a piece of tracing paper, and will find it to be in reality extremely simple.

Another simple plan is to gum the drawings of the sections on cardboard of a thickness proportional to the thickness of the section and the magnification, cut out all the cavities of the drawing with a knife or fret-saw, and gum all the fretwork thus obtained together. This gives, of course, a model of the object.

In simple cases it may be sufficient to adopt the plan described by SCHAFFER (*Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 342). Careful outlines of the sections to be reconstructed are drawn on tracing-paper with the aid of the camera lucida, superposed, and held up against the light for examination by transparence. EYCLESHEIMER'S method of producing orientation lines in celloidin imbedding mass has been described in Chap. XVI.

Mammalia.

588. Rabbit.—The rabbit may conveniently be taken as a type for this kind of work.

Dissection.—For the study of the early stages the ova must be sought for in the *tubæ* a certain number of hours after copulation. The dehiscence of the follicles takes place about ten hours after the first coitus. The *tubæ* and *cornua* having been dissected out should be allowed to cool, and remain until the muscular contractions have ceased. Then, with the aid of fine scissors or a good scalpel, all the folds of the genital duct are carefully freed from their peritoneal investment.

The tubæ are then (if the ova are still within them, which

is the case up to the end of the third day after coition) laid out on a long slip of glass, and slit up longitudinally by means of a pair of fine, sharp scissors. By means of needles and forceps the tubal mucosa is spread out so as to smooth out its folds as much as possible, and is carefully looked over with a strong lens or with a low power of the microscope. When the ova are found a drop of some "indifferent" liquid is dropped on each, and it is carefully taken up with the point of a scalpel, a cataract needle, or a small pipette. They may be examined in the peritoneal fluid of the mother if the animal has been killed, or in its aqueous humour, or in amniotic liquid, or in blood-serum, or in Kronecker's or other artificial serum.

If you have not been able to find the ova with the lens or the microscope, scrape off the epithelium of the tubal mucosa with a small scalpel, mix it with a little indifferent liquid, and look for the ova under the microscope by transmitted light.

Another method, employed by Kölliker, consists in injecting solution of Müller or weak osmic acid into the oviduct by means of a small syringe, and collecting the liquid that runs out in a series of watch-glasses, in which the ova can very easily be found by the microscope.

The same doe may be made to serve for two observations, at some hours' or days' interval. A longitudinal incision of 8 to 10 centimetres length is made on the median or a lateral line of the abdomen; an assistant keeps the intestines in their place; a ligature is placed at the base of one of the uterine cornua, beneath the neck, and a second ligature around the mesometrium and mesovarium. The ovary, the tuba, and the cornu of that side are then detached with scissors. The abdomen is then closed by means of a few sutures passing through the muscle-layers and the skin. The animals support the operation perfectly well, and the development of the ova of the opposite side is not in the least interfered with. When it is desired to study these the animal may be killed, or may be subjected to a second laparotomy if it be desired to preserve it for ulterior observations.

During the *fourth, fifth,* and *sixth* days after copulation the ova of the rabbit are free in the uterine cornua; they are easily visible to the naked eye, and may be extracted by the same manipulations as those of the tubes. After the sixth day they are at rest in the uterus, but have not yet contracted adhesions with the mucosa, so that they can still be extracted whole. At this stage the parts of the cornua where the ova

are lodged are easily distinguishable by their peculiar aspect, the ova forming eminences of the size of a pea. The cornua should be cut up transversely into as many segments as there are eminences, care being taken to have the ova in the centre of the segments. You then fix each segment by means of two pins on the bottom of a dissecting dish, with the mesometrial surface downwards and the ovular eminence upwards. The dissecting dish is then filled up with serum or liquid of Müller, or 0·1 per cent. solution of osmic acid, or Kleinenberg's picro-sulphuric acid, or nitric acid, or acetate of uranium solution. With a small scalpel a longitudinal incision is made on the surface of the ovular eminence, not passing deeper than the muscular layer; the underlying uterine mucosa is then gently dilacerated with two pairs of small forceps, and the ovum set free in the liquid.

From the moment the ova have become adherent to the uterine mucosa they can no longer be extracted whole. The embryo being always situated on the mesometrial surface, the ovular eminence is opened by a *crucial* incision, and the strip of mucosa to which the embryo remains adherent is fixed with pins on the bottom of the dish. Ed. v. Beneden (see *Arch. de Biol.*, v, fasc. iii, 1885, p. 378) has been able by operating in this way in serum of Kronecker, and keeping the whole at blood temperature, to observe the circulation of the embryo for hours together. (If this be desired to be done, the crucial incision should not be too extended, so as to leave the terminal sinus intact.)

Preparation.—In order to make permanent preparations of the different stages of fecundation and segmentation, v. Beneden (*Arch. de Biol.*, i, 1, 1880, p. 149) recommends the following process:—The living ovum is brought into a drop of 1 per cent. osmic acid on a slide, and thence into solution of Müller (or bichromate of ammonia or solution of Kleinenberg). After an hour the liquid is changed, and the whole is put into a moist chamber, where it remains for two or three days. It is then treated with glycerin of gradually increasing strength, and at last mounted in pure glycerin acidified with formic acid. Ova may be stained with Beale's carmine or picro-carmine, after removal from the osmic acid and careful washing.

In order to bring out the outlines of blastoderm-cells the

living ovum may be brought into one third per cent. solution of nitrate of silver. After remaining there for half a minute to two minutes, according to the age of the vesicle, it is brought into pure water and exposed to the light. The preparations thus obtained are instructive, but blacken rapidly, and cannot be permanently preserved.

After the end of the third day the blastodermic vesicle can be opened with fine needles, and the blastoderm washed, stained, and mounted in glycerin or balsam. v. Beneden has also obtained good preparations by means of chloride of gold.

For embryonic areas and more advanced embryos Kölliker recommends putting the ovum into 0·5 per cent. solution of osmic acid until it has taken on a somewhat dark tint, which happens in about an hour, and then treating it with successive alcohols for several hours. If the ovum be adherent to the uterine mucosa the portion of the membrane to which it is fixed should be left, stretched out with pins, in 0·1 per cent. solution of osmic acid for from four to six hours. The blastodermic vesicle can then easily be removed, and immersed for a few hours more in 0·5 per cent. solution of osmic acid, and finally be brought into alcohol. For sections Kölliker fixes with osmic acid. v. Beneden treats the ova for twenty-four hours with 1 per cent. solution of chromic acid, then washes well, and brings them through successive alcohols. Chromic acid has the advantage of hardening thoroughly the vesicle, and maintaining at the same time the epiblast-cells perfectly adherent to the zona pellucida. v. Beneden also recommends the liquid of Kleinenberg. Henneguy writes that he frequently employs it for embryonic areas and embryos of various ages, always with excellent results. Fol's modification of the liquid of Flemming, and Ranvier and Vignal's osmic acid and alcohol mixture (§ 28), also give excellent results. For staining, Henneguy recommends borax-carmine or Delafield's hæmatoxylin for small embryos; for large ones, Henneguy's acetic acid alum-carmine is the only reagent that will give a good stain in the mass.

For sections, pure paraffin. Cut in series and mount in balsam.

Piersol (*Zeit. f. wiss. Zool.*, xlvii, 2, 1888, p. 155) has been

lately using for fixation either Kleinenberg's solution or, for young stages, Altmann's 3 per cent. nitric acid. Staining and cutting as above.

Aves.

589. Superficial Examination.—Excellent instructions on this head are given in FOSTER and BALFOUR'S *Elements of Embryology*, to which, as it is certain to be in the student's hands, he may be referred. What follows here is given merely as being of more recent publication.

If it be desired to observe a living embryo by transmitted light, the egg should be opened under salt solution, as described below. A little of the white is then removed through the window, the egg is lifted out of the liquid, and a ring of gummed paper is placed on the yolk so as to surround the embryonic area. As soon as the paper adheres to the vitelline membrane, which will be in a few minutes, a circular incision is made in the blastoderm outside the paper ring. The egg is put back into the salt solution, and the paper ring removed, carrying with it the vitelline membrane and the blastoderm, which may then be brought into a watch-glass or on to a slide and examined under the microscope (DUVAL).

590. Gerlach's Window Method (*Nature*, 1886, p. 497; *Journ. Roy. Mic. Soc.*, 1886, p. 359).—Remove with scissors the shell from the small end of the egg; take out a little white by means of a pipette; the blastoderm will become placed underneath the window thus made, and the white that has been taken out may be replaced on it. Paint the margins of the window with gum mucilage, and build up on the gum a little circular wall of cotton wool; place on it a small watch-glass (or circular cover-glass), and ring it with gum. When the gum is dry, the cover is further fixed in its place by means of collodion and amber varnish, and the egg is put back in its normal position in the incubator. The progress of the development may be followed up to the fifth day through the window.

A description of further developments of this method, with figures of special apparatus, will be found in *Anat. Anz.*, ii, 1887, pp. 583, 609; see also *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 369.

591. Preparation.—During the first twenty-four hours of incubation it is extremely difficult to separate the blastoderm from the yolk, and they should be fixed and hardened together. In later stages, when the embryo is conspicuous, the blastoderm can easily be separated from the yolk, which is very advantageous. To open the egg, lay it on its side and break the shell at the broad end by means of a sharp rap; then carefully remove the shell bit by bit by breaking it away with forceps, working away from the broad end until the blastoderm is exposed. The egg should be opened in salt solution, then lifted up a little, so as to have the blastoderm above the surface of the liquid; the blastoderm is then treated with some fixing solution dropped on it from a pipette (1 per cent. solution of osmic acid, or Ranvier and Vignal's osmic acid and alcohol mixture, iodised serum, solution of Kleinenberg, 10 per cent. nitric acid, &c.). By keeping the upper end of the pipette closed, and the lower end in contact with the liquid on the blastoderm, the blastoderm may be kept well immersed for a few minutes, and should then be found to be sufficiently fixed to be excised. (Of course, if you prefer it, you can open the egg in a bath of any fixing liquid [10 per cent. nitric acid being convenient for this purpose] of such a depth as to cover the yolk; and having exposed the blastoderm, leave it till fixed [fifteen to twenty minutes]; but I think the procedure above described will generally be found more convenient.)

The egg is put back into the salt solution, and a circular incision made round the embryonic area. The blastoderm may then be floated out and got into a watch-glass, in which it may be examined, or may be brought into a hardening liquid.

Before putting it into the hardening fluid, the portion of vitelline membrane that covers the blastoderm should be removed with forceps and shaking.

In order to counteract the turning up of the edges of the blastoderm that generally happens during the process of hardening, it is well to get the blastoderm spread out on the *convex* surface of a watch-glass, and leave it so during the hardening.

For hardening, FOSTER and BALFOUR recommend solution of Kleinenberg for five hours, followed by alcohol. Or chromic

acid, a solution of 0·1 per cent. for twenty-four hours, followed by a solution of 0·3 per cent. for twenty-four hours more, then by 70 per cent. alcohol for a day, 90 per cent. alcohol for two days, and lastly absolute alcohol. They also recommend a 0·5 per cent. solution of osmic acid, in which the embryo remains for two hours and a half in the dark, and after washing is brought into absolute alcohol.

Henneguy prefers the osmic acid and alcohol mixture of Ranvier and Vignal, or Flemming's mixture followed by successive alcohols.

Staining and imbedding may be performed by the usual methods.

Up to about the fiftieth hour embryos may be mounted entire, in glycerin or balsam.

592. M. Duval's Orientation Method (*Ann. d. Sc. nat. Zool.*, 1885).—In the early stages of the development of the ova of Aves, before the appearance of the primitive streak, it is difficult to obtain a correct orientation of the hardened cicatricula, so as to be able to make sections in any desired direction. Duval, starting from the fact that during incubation the embryo is almost always found to be lying on the yolk in such a position that the big end of the egg is to the left, and the little end to the right of it, marks the position of the blastoderm in the following way.

With a strip of paper 5 millimetres wide and 50 millimetres long you construct a sort of triangular bottomless box. You lay this on the yolk, enclosing the cicatricula in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo, and its apex to the posterior region; that is to say, if the big end of the egg is to your left, the apex of the triangle will point towards you. You now, by means of a pipette, fill the paper triangle with 0·3 solution of osmic acid. As soon as the preparation begins to darken you put the whole egg into weak chromic acid, remove the white, and put the rest into clean chromic acid solution for several days. After hardening you will find on the surface of the yolk a black triangular area, which encloses the cicatricula and marks its position; you cut out this area with scissors and a scalpel, and complete the hardening with chromic acid and alcohol.

Another way of hardening is to place the egg, after the action of the osmic acid, in a solution of chromic acid which is then raised to boiling-point on a water-bath; after cooling, the blackened region is cut out, and the hardening completed in the usual way with chromic acid and alcohol.

593. Köller's **Method** (*Arch. f. mik. Anat.*, xx, 1881, p. 182).—Chromic acid, 0·1 per cent., twenty-four hours; *idem*, 0·2 per cent., twenty-four hours; and so forth, with daily increments of 0·1 per cent. up to 0·5 per cent. When hard, remove the blastoderm together with a segment of the yolk. Water, twenty-four hours. Stain and imbed.

594. Vialleton's **Method** (*Anat. Anzeig.*, vii, 1892, pp. 624—627; *Journ. Roy. Mic. Soc.*, 1892, p. 889).—Egg opened in salt solution, blastoderm excised and removed to a glass plate, then treated with 1 per cent. nitrate of silver solution, washed with water, and put into 70 per cent. alcohol for six to twelve hours in the dark. Borax-carmine, alcohol, dammar.

Reptilia.

595. General Directions.—The methods described above for the embryology of birds are applicable to the embryology of reptiles. During the early stages the blastoderm should be hardened *in situ* on the yolk; later the embryo can be isolated, and treated separately with Kleinenberg's solution and alcohol (Strahl, *Arch. f. Anat. u. Phys.*, 1881, p. 123).

596. Kupffer's **Method** (*ibid.*, 1882, p. 4).—The ova are opened and the albumen removed under osmic acid of $\frac{1}{10}$ per cent. The yolk is put for twenty-four hours into an ample quantity of $\frac{1}{3}$ per cent. chromic acid solution; the blastoderm is removed, washed out in water, and put for three hours into Calberla's liquid (āā glycerin, water, and alcohol), and finally hardened in 90 per cent. alcohol.

597. Sarasin's **Method** (Semper's *Arbeiten*, 1883, p. 159).—Fix with chromic acid or hot water, and harden with alcohol. Stain with Bismarck brown, alum-carmine, or hæmatoxylin, or picro-carmine. Imbed in collodion, and collodionise the sections as cut.

Amphibia.

598. Preliminary.—In order to prepare the ova of Amphibia for section cutting, it is essential to begin by removing their thick coats of albumen. This may be done by putting them for two or three days into 1 per cent. solution of chromic acid, and shaking well; but ova thus treated are very brittle, and do not afford good sections. A better method is that described by Whitman (*Amer. Natural.*, xxii, 1888, p. 857), and by Blochmann (*Zool. Anz.*, 1889, p. 269). Whitman puts the fixed eggs into a 10 per cent. solution of sodium hypochlorite

diluted with 5 to 6 volumes of water, and leaves them there till they can be shaken free, which happens (for *Necturus*) in a few minutes. Blochmann takes *eau de Javelle* (potassium hypochlorite), and dilutes it with 3 to 4 volumes of water, and agitates the eggs, previously fixed with solution of Flemming, for fifteen to thirty minutes in it. The ova are afterwards preserved in alcohol in the usual way. Some other means of attaining the same end are given in the following paragraphs.

599. Axolotl.—The ova are easier to prepare than those of the Anura, because the yolk is separated from the albuminous layer by a wide space filled with a liquid that is not coagulated by reagents. Put the eggs for a few hours into picro-sulphuric acid, then pierce the inner chorion with fine scissors or needles, and gently press out the ovum. Harden in alcohol.

600. Triton (Scott and Osborn, *Quart. Journ. Mic. Sci.*, 1879, p. 449).—The albumen is here present in the form of several concentric coats, which are very delicate. Incise each of them separately with fine scissors, turn out the ovum, and fix it. Solution of Kleinenberg is the reagent that gives the best results.

601. Triton (Hertwig, *Jen. Zeit. f. Naturw.*, 1881–2, p. 291).—Put the eggs into a mixture of equal parts of 2 per cent. acetic acid and 0·5 per cent chromic acid. After ten hours incise the membranes, opening one end of the inner chorion, and turn out the embryos and bring them into successive alcohols.

602. Salamandra (Rabl, *Morphol. Jahrb.*, xii, 2, 1886, p. 252).—Fix in chloride of platinum of 0·25 to 0·3 per cent., kept warm for from three to twenty-four hours, according to the size of the embryos, wash well with water, treat with successive alcohols, make sections, and stain on the slide.

603. Rana (O. Hertwig, *Jen. Zeit. f. Naturw.*, xvi, 1883, p. 249).—The ova are thrown into nearly boiling water (90° to 96° C.) for five to ten minutes. The albuminous envelope of the ovum is then cut open, and the ovum extracted under water. The ova are then brought into 0·5 per cent. osmic acid, or into alcohol of 70, 80, and 90 per cent. Chromic acid

makes ova brittle; they ought not to remain in it for more than twelve hours. Chromic acid destroys or attacks the pigment of the ova, whilst alcohol preserves it, which is frequently important for the study of the germinal layers.

Morgan (*Amer. Nat.*, xxv, 1891, p. 759; *Journ. Roy. Mic. Soc.*, 1892, p. 284) has the following. During the periods in which it is difficult or impossible to remove the inner jelly-membrane the eggs can be freed as follows:—Each egg is cut out with scissors from the general jelly-mass, and put for from one to twelve hours into saturated solution of picric acid in 35 per cent. alcohol containing "the same amount of sulphuric acid as in Kleinenberg's solution." Wash for several hours in several changes of alcohol, beginning with 35 per cent., and increasing the strength gradually up to 70 per cent. About the second day in the 70 per cent. alcohol the inner membrane begins to swell, and on the third or fourth day may be pierced by a needle, and the egg removed and placed in 80 per cent. alcohol (see also Whitman, *Meth. of Research*, p. 156; and Schultze, *Zeit. f. wiss. Zool.*, v, 1887, p. 177).

Pisces.

604. Teleostea in General.—The ova of many of the bony fishes can be studied by transmitted light in the living state; but those of the Salmonidæ must be hardened and removed from their envelopes for the study of the external forms of the embryo.

To this end the ova may be put for a few minutes into water containing 1 to 2 per cent. of acetic acid, and thence into 1 per cent. chromic acid. After three days the capsule of the ovum may be opened at the side opposite to the embryo, and be removed with fine forceps. The ovum is put for twenty-four hours into distilled water, and then into successive alcohols. Embryos thus prepared show no deformation, and their histological elements are fairly well preserved. But the vitellus rapidly becomes excessively hard and brittle, so as greatly to interfere with section cutting.

The following processes give good results as regards section cutting.

Put the ova for a few minutes into 1 per cent. osmic acid; as soon as they have taken on a light brown colour bring them

into Müller's solution. Open them therein with fine scissors—the vitellus, which immediately coagulates on contact with air, dissolves, on the contrary, in Müller's solution—and the germ and cortical layer can be extracted from the capsule of the ovum. They should be left in clean Müller's solution for a few days, then washed with water for twenty-four hours, and brought through successive alcohols.

Another method is as follows :—The ova are fixed in solution of Kleinenberg containing 10 per cent. of acetic acid. After ten minutes they are opened in water containing 10 per cent. of acetic acid, which dissolves the vitellus. The embryos are put for a few hours into pure solution of Kleinenberg, and are then brought through alcohol of gradually increasing strength.

605. Kollmann's **Method** (see § 40).

606. Perényi's **Method** (see § 39).

607. Kowalewsky's **Method** (*Zeit. f. wiss. Zool.*, xliii, 1886, p. 434).—Fix for an hour and a quarter in a mixture of 8 volumes of picro-sulphuric acid with one of 1 per cent. chromic acid. Wash out for twelve hours with 20 per cent. alcohol, and pass the ova very gradually through alcohol of 20, 28, 35, 43, 50, 60, and 70 per cent. strength, the last to be changed frequently until all the picric acid is extracted. Before staining, the capsules of the ova should be opened. Stain with borax-carmine or hæmatoxylin, and imbed in paraffin.

608. Boyer's **Methods.**—See *Bull. Mus. Comp. Zool.*, Harvard, xxiii, 1892, p. 93 ; *Journ. Roy. Mic. Soc.*, 1892, p. 699.

609. Rabl-Rückhard's **Method** (*Arch. f. Anat. u. Entw.*, 1882, p. 67).—Fix in 10 per cent. nitric acid for fifteen minutes. Remove the membranes to avoid deformation of the embryos, and put the ova back into the acid for an hour. Wash out in 1 to 2 per cent. solution of alum for an hour, and harden in alcohol.

Modification of this method by Goronowitsch (see *Morph. Jahrb.*, x, 1884, p. 381).

610. Pelagic Fish Ova (Whitman's method ; *Amer. Natural.*, xvii, 1883, pp. 1204–5 ; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 912, and *Methods of Research*, &c., p. 152).—Fix by treatment first for five to ten minutes with a mixture of equal parts of sea water and $\frac{1}{2}$ per cent. osmic acid solution, and then for one or two days with a modified Merkel's solution (due to Eisig), consisting of equal parts of 0·25 per cent. platinum chloride and 1 per cent. chromic acid. Prick the membrane before transferring to alcohol. Whitman found that the usual Merkel's fluid caused maceration of the embryonic portion of the egg. Picro-sulphuric acid causes the embryonic cells to swell, and in many cases to become completely disorganised. The

osmic acid treatment is necessary in the case of segmenting ova because the Merkel's fluid does not kill rapidly enough, so that eggs placed in it may even pass through one or two stages of cleavage before dying. This fluid arrests the process of blackening by the osmium, or will even bleach the objects if blackening has set in. See also AGASSIZ and WHITMAN, in *Proc. Amer. Acad. Arts and Sciences*, xx, 1884. For later stages the authors recommend the method of Perényi. And see the experiments detailed by COLLINGE, *Ann. and Mag. Nat. Hist.*, x, 1892, p. 228; *Journ. Roy. Mic. Soc.*, 1892, p. 883.

Tunicata.

611. Distaplia.—DAVIDOFF (*Mitth. Zool. Stat. Neapel*, ix, 1, 1889, p. 118) has some important observations on the fixation of the ova of *D. magnilarva*. The best reagent is a mixture of 3 parts of saturated solution of corrosive sublimate and 1 of glacial acetic acid. The ova to remain in it for from half an hour to an hour, and be then washed for a few minutes in water and brought through successive alcohols. Another reagent, almost as good, consists of 3 parts of saturated solution of picric acid and one of glacial acetic acid, the objects to remain in it for three to four hours, and then be brought into 70 per cent. alcohol.

612. Amarœcium (MAURICE and SCHULGIN, *Ann. Sci. Nat. Zool.*, xvii, 1884).—Stain in borax-carmine, wash out, and stain for fifteen to twenty hours in very weak solution of *bleu de Lyon* in 70 per cent. alcohol with a few drops of acetic acid. In sections the epiblast and hypoblast appear chiefly blue, the mesoblast-cells, on the contrary, appearing almost entirely red.

Mollusca.

613. Cephalopoda (USSOW, *Arch. de Biol.*, ii, 1881, p. 582).—Segmenting ova are placed, without removal of the membranes, in 2 per cent. solution of chromic acid for two minutes, and then in distilled water, to which a little acetic acid (one drop to a watch-glassful) has been added, for two minutes. If an incision be now made into the egg-membrane the yolk flows away and the blastoderm remains; if any yolk still cling to it, it may be removed by pouring away the water and adding more.

WATASE (*Journ. of Morphol.*, iv, 1891, p. 249; *Journ. Roy. Mic. Soc.*, 1892, p. 152) kills the ova in the macerating mix-

ture of the Hertwigs (§ 542), and as soon as the blastoderm turns white and opaque removes it under dilute glycerin. Treatment with liquid of Perényi is recommended for surface views of cleavage.

614. Gastropoda (Henneguy).—Ova of Helix may be fixed for from four to six hours in Mayer's picro-nitric solution (§ 58). The carbonate of lime that encrusts the external membrane is thus dissolved, and the albuminous coat of the egg is coagulated. The egg is opened with needles, the albumen comes away in bits, and the embryo can be removed. Treat with successive alcohols, and imbed in paraffin.

615. Limax (early stages) (Mark, *Bull. Mus. Comp. Zool., Harvard Coll.*, vi, 1881).—The ova are treated with acetic acid of 1 to 2 per cent. for four or more hours. The two external membranes are incised with fine scissors, and the egg squeezed out in its albumen membrane. This is dissected off on a slide, the egg is separated from the albumen, stained, and mounted in glycerin.

For later stages, or for making sections, osmic acid is used instead of acetic acid, and the egg is hardened within its albuminous coats.

Henchman (*Bull. Mus. Comp. Zool., Harvard,* xx, 1890, p. 171; *Journ. Roy. Mic. Soc.*, 1891, p. 274; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 216) finds that the best way of obtaining embryos is to keep some twenty-five or thirty adults (of *L. maximus*) in a large tin pail with a cover perforated with small holes. They should be fed on cabbage, and the vessel kept very clean. Eggs are generally found in the morning in bunches of thirty to forty. As they are more abundant in the early stages of confinement it is better to obtain a few slugs often than many at once. In a moderately warm room hatching occurs between the twenty-second and twenty-seventh day. The eggs must be carefully protected from desiccation.

Kill with 0·33 per cent. chromic acid, or with liquid of Perényi. It is best to remove only the outer envelope before putting into the chromic acid, the inner membrane being removed after two or three minutes therein. Where Perényi is used the membranes must be removed first, as the albumen will else coagulate in such a way as to prevent the removal of the embryos.

See also Schmidt, *Studien zur Entwickelungsgesch. d. Pulmonaten,* Dorpat, 1891.

Arthropoda.

616. Fixation of Ova.—In most cases the ova of Arthropods are fixed by heat in a more satisfactory way than by any other means. This may be followed either by alcohol or some watery hardening agent. If it be desired to avoid heating, picro-sulphuric acid or liquid of Perényi may be tried.

Removal of Membranes.—This is frequently very difficult, and it may often be advisable not to attempt to remove them, but to soften them with *eau de Javelle* or *eau de Labarraque* (see the methods of Looss and List).

Morgan (*Amer. Natural.*, xxii, 1888, p. 357; *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 69) recommends (for the ova of *Periplaneta*) *eau de Labarraque* diluted with five to eight volumes of water, and slightly warmed. Thus used, it will soften the chitin membranes sufficiently in thirty to sixty minutes if employed before fixing. Fixed ova take longer. The fluid must, of course, not be allowed to penetrate into the interior of the ovum.

617. Henking's **Methods.**—For the whole subject of the technique of the embryology of Insecta see an elaborate paper by this specialist in *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 156. Henking agrees with other workers at this subject, that in the majority of cases heat is the only available fixing agent that will give fair results. He kills ova by plunging them into hot water, or by pouring hot water on to them in a watch-glass, and then removing into 70 per cent. alcohol. But, as might be expected, he finds that the preservation of structures by this method is far from being perfectly satisfactory, cell-contours being not at all sharply brought out by it, and achromatic cell-structures being but imperfectly preserved. He finds that in some cases ova may be fixed with liquid of Flemming, which, as may be supposed, gives incomparably better results in these respects. Suitable ova may be put into liquid of Flemming (Henking does not say which formula) for half an hour, then for two hours into the same diluted with three volumes of water, then treated with alcohol as usual. Boveri's picro-acetic acid was found not to penetrate the membranes.

Henking thinks that eau de Javelle for softening mem-

branes is best avoided. Membranes should either be dissected away or left *in situ*, and cut with the rest of the egg, according to the nature of the case. The great obstacle to section cutting is the brittleness of the yolk. This difficulty may be overcome as follows :—After fixing and treating with alcohol, prick the chorion and stain with borax-carmine. Put the stained ova for twelve hours into a mixture containing 20 c.c. of 70 per cent. alcohol, one drop of concentrated hydrochloric acid, and a knife-pointful of pepsin (it is not necessary that all the pepsin should be dissolved). The ova may then be treated with alcohol, oil of bergamot, and paraffin, and (with some exceptions, amongst which is *Bombyx mori*) will be found to cut without crumbling.

The contents of fresh ova may conveniently be studied by means of the following fluid :

Distilled water	80 c.c.
Glycerin	16 ,,
Formic acid	3 ,,
1 per cent. osmic acid . . .	1 ,,
Dahlia	0·04 grm.

The eggs are simply teased in a drop of the liquid, and a cover-glass put on. If it be desired to preserve the preparation, nothing more is necessary than to lute the cover-glass.

618. Lepidoptera (Bobretzky, *Zeit. f. wiss. Zool.*, 1879, p. 198).—Ova (of *Pieris cratægi* and *Porthesia chrysorrhœa*) are slightly warmed in water and put for sixteen to twenty hours into 0·5 per cent. chromic acid. The membranes can then be removed, and the ova brought for a few hours into absolute alcohol, stained with carmine, and cut.

619. Blattida (Patten, *Quart. Journ. Mic. Sci.*, 1884, p. 549). —The ova or larvæ are placed in cold water, which is gradually raised to 80° C. You leave off heating as soon as the ova have become hard and white. Pass very gradually through successive alcohols, beginning with 20 per cent.; stain with Kleinenberg's hæmatoxylin or Mayer's cochineal (only alcoholic stains will traverse the chorion). The ova may remain in the hæmatoxylin for five or six days, and be washed out in alcohol containing one drop of HCl per 20 grms., in which they should remain for several days, and then

be soaked in pure alcohol until they have regained their violet colour. Penetrate with benzol and imbed in paraffin.

Wheeler (*Journ. of Morph.*, iii, 1889, p. 292; *Journ. Roy. Mic. Soc.*, 1890, p. 250) dissects out ovarian ova in salt solution and fixes in liquid of Perényi (fifteen minutes), then treats with alcohol, and stains with borax-carmine. Laid eggs may be killed by Patten's method. After heating, the two lips of the crista of the capsule may be separated with fine forceps and pieces of the walls torn away, and the eggs pushed out of the compartments formed by their choria and hardened as desired. Good results are also obtained by heating to 80° C. for ten minutes in liquid of Kleinenberg, and preserving in 70 per cent. alcohol. This causes the envelopes to dilate and stand off from the surface of the egg, so that they can easily be dissected away.

Cholodkowsky (*Mem. Acad. Imp. St. Petersburg*, xxxviii, 1891; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 80) recommends cutting off the ends of the cocoons and fixing for twelve hours in liquid of Perényi, or for a few minutes in a solution of 1 part iodine, 1 part iodide of potassium, and 300 parts water, heated to boiling-point.

Heymons (*Zeit. f. wiss. Zool.*, liii, 1892, p. 434; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 343) finds that Cholodkowsky's methods are good for the study of general relations of parts, but not satisfactory for the preservation of delicate detail. For young embryos it is better to incise the cocoon at the end by which it inheres in the body of the mother, bring it for two minutes into water heated to 90° C., and open in Flemming, in which the embryo should be dissected out.

620. Diptera (Henking, *Zeit. f. wiss. Zool.*, xlvi, 1888, p. 289; *Zeit. f. wiss. Mik.*, 1889, p. 59).—Ova still contained within the fly may be fixed by plunging the animal for some time into boiling water, then dissecting out and bringing them into 70 per cent. alcohol. Laid eggs may have boiling water poured over them, or be put into solution of Flemming in a test-tube which is plunged into boiling water until the eggs begin to darken (about a minute). Cold solution of Flemming easily causes a certain vacuolisation of the contents of the ova. Open the ova at the larger end, stain with borax-carmine for fifteen to thirty hours, and cut in paraffin.

See also (for *Chironomus*) Ritter, *Zeit. f. wiss. Zool.*, L, 1890, p. 408; *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 87 (strings of ova fixed with hot 30 per cent. alcohol containing a little sublimate, and stained in the mass by immersion for several days in picro-carmine).

621. Aphides (Will, *Semper's Arbeiten*, 1883, p. 223).—Sections to be made through the entire animals containing the ova and embryos. The animals are killed in water of 70° C., and brought into alcohol. The cuticle may then be pricked with a needle, and the animals stained in the mass with borax-carmine or hæmatoxylin. You may imbed in collodion and collodionise the sections as cut.

622. Araneina (Balfour, *Quart. Journ. Mic. Sci.*, 1880, p. 167).—Balfour hardened the embryos in bichromate of potash, after placing them for a short time in nearly boiling water. After removal of the membranes they were stained as a whole with hæmatoxylin.

Kishinouye (*Journ. Coll. Sci. Imp. Univ. Japan*, iv, 1891, p. 55; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 215) fixes in water warmed to 70° or 80° C., puts into 70 per cent. alcohol, and after twenty-four hours therein pierces the membranes and passes through stronger alcohol. Stain with alcoholic cochineal or picro-carmine, and imbed in paraffin.

See also Locy, *Bull. Mus. Comp. Zool., Harvard*, xii, 3, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 242. Fix by hot water. The liquid of Perényi may also be used; it has the advantage of not making the yolk so granular.

623. Phalangida (Balbiani).—The ova of *Phalangium opilio* are enclosed in a chorion covered with yellow corpuscles which renders them quite opaque. They may be cleared by treating them with water containing a little solution of caustic potash and raised to boiling-point. The ova are then laid on blotting-paper, and the chorion is removed by rubbing them gently with a small brush. The vitelline membrane remains intact and transparent, and the embryo may be studied through it.

624. Phalangida (Henking, *Zeit. f. wiss. Mik.*, iii, 4, 1886, pp. 470 *et seq.*).—Fix with boiling water or "Flemming." Preserve the ova in 90 per cent. alcohol. To open the chorion, bring them back into 70 per cent. alcohol, which causes them to swell up so that the chorion can easily be pierced with needles, and the ovum turned out.

625. Astacus (Reichenbach, from *Zeit. f. wiss. Mik.*, 1886, p. 400).—Fix in water gradually warmed to 60° or 70° C. (if the chorion should burst, that is no evil), harden for twenty-four hours in 1 to 2 per cent. bichromate of potash or 0·5 per cent. chromic acid, wash out for the same time in running water, and bring into alcohol. Remove the chorion, remove the embryo from the yolk by means of a sharp knife, and stain with picro-carmine and mount in balsam.

626. Amphipoda (*Orchestia*) (Ulianin, *Zeit. f. wiss. Zool.*, xxxv, 1881, p. 441).—Ova in the earliest stages of development were treated for two hours with picro-sulphuric acid (Kleinenberg's formula). This causes the chorion to swell and burst. Ova in later stages, in which the embryo is surrounded by a cuticular membrane, which encloses an albuminous liquid, must have this membrane torn with needles and the albuminous liquid allowed to ooze out before placing in the picro-sulphuric acid.

627. Maturation of Ova, and other early stages.—These should be studied by the methods given in the chapter on "Cytological Methods."

Vermes.

628. Tænia (v. Beneden, *Arch. de Biol.*, ii, 1881, p. 187).—Ova in which a chitinous membrane has formed around the embryo are impervious to reagents. They may be put on a slide with a drop of some liquid and covered. Then, by withdrawing the liquid by means of blotting-paper, the cover may be made to gradually press on them so as to burst the membranes, and the embryo may then be treated with the usual reagents.

629. Planaria (Iijima, *Zeit. f. wiss. Zool.*, xl, 1884, p. 359).—The capsule containing the ova (of fresh-water Planaria) is opened with needles on a slide, in a drop of 2 per cent. nitric acid. The ova are extracted and covered (the cover being supported by paper, or by wax feet). After half an hour they are treated with successive alcohols under the cover, and finally mounted in glycerin. For sections, the whole of the contents of a capsule is hardened in the mass in 1 per cent. chromic acid and cut together.

630. Lumbricus (Kleinenberg, *Quart. Journ. Mic. Sci.*, 1879, p. 207).—Fix with Kleinenberg's picro-sulphuric acid, or, which is not quite so good, with vapours of osmium, pass through successive alcohols, stain with Kleinenberg's hæmatoxylin, and cut in paraffin.

Wilson (*Journ. of Morph.*, iii, 1889, p. 445; *Journ. Roy. Mic. Soc.*, 1890, p. 402) finds that liquid of Perényi is by far the best fixing reagent, being in most respects superior even to Flemming's. Fix for fifteen to sixty minutes, wash out in 70 per cent. alcohol, stain with borax-carmine, wash out in acid alcohol, treat for a few minutes with ammoniacal alcohol to neutralise the acid, and after-stain for twelve hours with

very dilute Kleinenberg's hæmatoxylin. Imbed in paraffin, giving a short paraffin bath (not more than ten to fifteen minutes).

For study of entire specimens in the early stages, Perényi's liquid, followed by alcohol, water, very dilute iodine solution, and glycerin, gives the best results.

631. Ascaris.—See the chapter on "Cytological Methods."

Echinodermata, Cœlenterata, and Porifera.

See the paragraphs treating of these groups in Chap. XXXII.

For the maturation and fecundation of the ova of the Echinodermata, see also the chapter on "Cytological Methods."

CHAPTER XXVI.

CYTOLOGICAL METHODS.

632. The Methods of Study.—There are three ways of obtaining knowledge of cell-structure—study of living cells, study of fresh unhardened cells, and study of hardened material in sections. Of these the last is the most fruitful; and I advise the beginner to keep as close as possible to the method of Flemming and Rabl, utilising unhardened material chiefly for the purpose of controlling the results obtained by the study of sections, and reserving the study of living cells chiefly for establishing the seriation of already observed phenomena.

633. Subjects for Study.—One of the best objects for this purpose is the tail of young larvæ of Amphibia, both Anura and Urodela.

In the *living animal* the epithelial cells and nuclei (in the state of repose) are so transparent as to be invisible in the natural state. They may, however, be brought out by curarising the larva; or, still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare.—Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless; as soon as their movements have become slow they may be taken out and placed on a slide with blotting-paper. If they be replaced in water they return to the normal state in eight or ten hours, and may be re-curarised several times.

Etherisation.—Three per cent. alcohol, or 3 per cent. ether, may be used in a similar way. These reagents cause no obstruction to the processes of cell-division, and are useful, but their action as anæsthetics is inconstant.

Indifferent Media.—One per cent. salt solution, iodised serum, syrup, cold water (+1° C.), and warm water (35°—40° C.). The tail may be excised from the living animal and studied for a long time in these media (Peremeschko, *Arch. f. mik. Anat.*, xvi, 1879, p. 437).

Perhaps (Flemming, *ibid.*, pp. 304 *et seq.*) the very best subject for these studies is *Salamandra*. The adult offers for study the thin transparent bladder; in the larva the gills and caudal "fin" may be studied in the living state. The gills are difficult to fix in position for observation, and are obscured by pigment. In the fin there is always a spot, near to the hind limbs, that is free from pigment; and on lightly coloured larvæ other such spots may be found on the ventral half of the fin and on the lateral line. On a flat-finned larva it is possible to study these spots with high-power glasses.

The larva may be fixed in a suitable cell, or wrapped in moist blotting-paper, or may be curarised; or the tail may be excised. (It is preferable to cut through the larva close in *front* of the hind limbs.)

A favorable object for *preparation* is found in the gill-*plates*, delicate laminæ that are to be found attached to the gill-cartilages on the mouth side.

The lungs, parietal peritoneum, and mesentery of the larvæ are also very favorable objects for preparations (see Flemming, *Arch. f. mik. Anat.*, xxxv, 1890, p. 275; and xxxvii, 1891, pp. 249 and 685). To prepare the lungs the larva, which should be of not more than 4 cm. length, should be killed by immersion in chromo-aceto-osmic acid, the body-cavity cut into, and the viscera gently drawn out and exposed to the action of the liquid, care being taken not to let the lungs get into folds. After fixation they should be carefully got on to a slide, and a small strip removed from their margins on either side by means of a scalpel, after which the two walls may be separated from each other, and utilised as thin, flat preparations.

Another excellent object is the intestine of the adult, of which sections may be made by the paraffin method, as recom-

mended in the important paper of M. HEIDENHAIN, "Über Kern und Protoplasma," in *Festschr. z. 50 jähr. Jubil. d. H. Prof. Geheimr. v. Kölliker* (also in separate reprint), Engelmann, Leipzig, 1892, p. 111. This organ offers for study, besides the large epithelium-cells of the intestinal crypts, numberless examples of leucocytes, an extremely favorable object.

Larvæ may be bred from adults kept in confinement, and supplied with a vessel of water, in which they will place the larvæ of their own accord. In May gravid females may be killed and the larvæ extracted. The larvæ must be kept in frequently changed water and fed every day or two. Aquatic worms may be used for feeding them, *e. g. Tubifex rivulorum.*

It is extremely important that they should be fed regularly and abundantly, for, if not, cell-divisions in the tissues become rare, and may even cease altogether.

Other classical subjects of study will be found mentioned in the following paragraphs.

634. Stains for Living Cells.—It is sometimes of the very greatest importance to be able to stain a cell in the living state, even though it be but feebly and imperfectly. Methylen blue, dahlia, or gentian violet may be used in solution in pure water, or in an indifferent liquid; the addition of a trace of chloral hydrate will enable you to obtain a clear solution of the last two in saline media. It is sometimes advisable to rub them up with serum, as recommended by V. LA VALETTE ST. GEORGE. These methods are most important for the study of the *Nebenkern.*

The student will remember that no known reagent will stain any part of the nucleus whilst alive. The "Nebenkern" stains sometimes, but feebly. Most frequently the colour is only taken up by certain granules of the cytoplasm, which may or may not be identical with the "granules" or "bioblasts" of ALTMANN.

These matters have already been discussed in the paragraph headed "*Staining 'intra vitam,'*" § 93.

635. Study of Fresh and Lightly fixed Cells.—It has been rightly pointed out by Flemming that so-called "indifferent" liquids must not be believed to be without action on nuclei. Iodised serum, salt-solution, serum, aqueous humour, lymph, better deserve the name of weak hardening agents. Between

these, and such energetic hardening agents as Flemming's mixture, come such light fixing agents as picric acid or very dilute acetic acid. These it is whose employment is indicated for the study of fresh isolated cells.

A typical example of this kind of work is as follows :—Tease out a piece of living tissue in a drop of acidulated solution of methyl green (0·75 per cent. of acetic acid). This is a delicate fixing agent, killing cells instantly without change of form. Complete the fixation by exposing the preparation for a quarter of an hour to vapour of osmium, and add a drop of solution of Ripart and Petit and a cover.

Or you may fix the preparation, after teasing, with vapour of osmium for half a minute to two minutes, then add a drop of methyl green, and after five minutes wash out with 1 per cent. acetic acid, and add solution of Ripart and Petit and cover.

Or you may kill and fix the cells by teasing in solution of Ripart and Petit (to which you may add a trace of osmium if you like), and afterwards stain with methyl green.

Henking's mixture, which has been given above (§ 617), may also be found useful.

Flemming (*Arch. f. mik. Anat.*, xx, 1881, p. 3) found the following method useful for the study of the division of the ova of Echinodermata :—The ova are stained on the slide by adding the stain at the edge of the cover. Safranin or other nitro- or anilin colours may be used. As soon as the entire ovum is of a dark colour the stain is drawn off with blotting-paper, and acetic acid of 1 per cent. added. Schneider's acetic carmine (*Zool. Anzeig.*, 1880) (see § 158) is very convenient, and gives good results. (For the details of the manipulation by which these reagents are added and drawn off on the slide it is well to consult the article quoted, p. 6.)

Another good method is as follows :—Segmenting ova are treated with a mixture of 40 to 50 parts of concentrated nitric acid with 60 to 50 parts water. Wash with water until all the yellow stain of the nitric acid has disappeared; stain with Schneider's acetic carmine, and mount in glycerin. (The preparations cannot be said to be permanent, as after a time the stain darkens in such a way as to render the nuclear figures unrecognisable.)

Other fixing agents, such as picric acid or weak sublimate

solution, may of course be used, and in some cases doubtless should be preferred. Other stains, too, such as Bismarck brown or Delafield's hæmatoxylin, may be used as occasion dictates; and of course other examination media than solution of Ripart may be employed. But, for general purposes, the methyl-green-osmium-and-Ripart's-medium method gives such good results, and is so very convenient, that it may well be called the classical method for the study of fresh cells. I think great credit is due to CARNOY for his frequent insistence on the excellence and handiness of this method.

Other fixing agents and stains that are applicable to this kind of work will be found discussed in the course of the following paragraphs.

636. Some Microchemical Reactions.—Methyl green is a test for chromatin, in so far as it colours nothing but the chromatin *in the nucleus*. It is, however, not a perfect test, for the intensity of the coloration it produces varies greatly in different nuclei, and may in certain nuclei be extremely weak, or (apparently) even altogether wanting. In these cases other tests must be applied in order to establish with certainty the presence or absence of that element. The following suggestions are taken from CARNOY, who is, I believe, the only writer—on the zoological side, at all events—who has insisted on the necessity of applying microchemical methods in a systematic manner to the study of cells.

Chromatin is distinguished from the lecithins and from albuminoids by not being soluble, as these are, in water and in weak mineral acids, such as 0·1 per cent. hydrochloric acid. It is easily soluble in concentrated mineral acids, in alkalies, even when very dilute, and in some alkaline salts, such as carbonate of potash and biphosphate of soda. In the presence of 10 per cent. solution of sodium chloride it swells up into a gelatinous mass, or even, as frequently happens, dissolves entirely (*Biol. Cell.*, pp. 208–9). It is only partially digestible (when *in situ* in the nucleus) in the usual laboratory digestion fluids.

The solvents of chromatin that are the most useful in practice are 1 per cent. caustic potash, fuming hydrochloric acid, or cyanide of potassium, or carbonate of potash. These last generally give better results than dilute alkalies. They may be

employed in solutions of 40 to 50 per cent. strength. If it be desired to remove all the chromatin from a nucleus the reaction must be prolonged, sometimes to as much as two or three days, especially if the operation be conducted on a slide and under a cover-glass, which is the safer plan.

It must be remembered that these operations must be performed on *fresh* cells, for hardening agents bring about very considerable modifications in the nature of chromatin, rendering it almost insoluble in ammonia, potash, or sodic phosphate, &c. Hydrochloric acid, however, still swells and dissolves it, though with difficulty.

Partial digestion may render service in the study of the chromatic elements of nuclei. Chromatin resists the action of digestive fluids much longer than the albumens do; so that a moderate digestion serves to free the chromosomes from any caryoplasmic granulations that may obscure them, whilst at the same time it clears up the cytoplasm.

In the last edition the term "nuclein" was used throughout this section in all the places where the term "chromatin" has been used in the above paragraphs. It is now known that there exists a whole series of nucleins, differing chiefly in respect of their richness in phosphorus and proteids. At one end of the chain is nucleic acid, with 9 to 11 per cent. of phosphorus, and without any proteid (this compound occurs in nature in the heads of spermatozoa); in the middle are what are generally termed the nucleins, consisting of proteid with varying amounts of nucleic acid; and at the other extreme are nucleins which are nearly all proteid, containing only 0·5 to 1 per cent. of phosphorus, and are in fact the same substances which have received the name of "nucleo-albumin;" they may also be termed the artificial plastins.

These substances have both been isolated from the most diverse tissues of the animal body, and have been prepared artificially. A corresponding series of nucleins exists within the nucleus itself. There are those that contain most nucleic acid; these are readily soluble in alkalies, and precipitable with difficulty by acid: chromatin is one of these. There are others more insoluble in alkalies and poorer in nucleic acid: these are the plastins, the pyrenin of nucleoli being one of them. And there are others even poorer in nucleic acid: these are the nucleo-albumins (which exist also in the cytoplasm); the paralinin, or nuclear sap, appears to be in part composed of these, in part of phosphorus-free compounds.

There appears to be some doubt whether chromatin is or is not nucleic acid itself. The principal reactions in which it resembles nucleic acid are given by Halliburton (*Goulstonian Lectures on the Chemical Physiology of the Animal Cell*, 1893, p. 574 of the Report in the *British Medical Journal*, No. 1681, March 18th, 1893, from which place also I have condensed the above remarks on the chemistry of the nucleins) as follows:—

"1. It does not give Millon's nor the xantho-proteic reactions. 2. It is easily soluble in alkalies, soluble with difficulty in acids. 3. It is soluble in an acetic acid solution of potassium ferrocyanide. 4. After treatment with concentrated copper sulphate solution for twenty-four hours it loses its affinity for stains. It is not, however, dissolved by the copper sulphate as Schwartz stated. 5. It has a great affinity for anilin dyes, especially for basic dyes like methyl green. If a mixture of methyl green and acid fuchsin is employed, nucleic acid is stained green. The nucleins next richest in phosphorus are stained a blue-violet tint, whereas the phosphorus-poorest are coloured red. Now in the dividing nucleus, when the amount of chromatin is at its maximum, the nucleus stains green; whereas in the resting nucleus, where there is more pyrenin, a blue colour is observed."

These considerations appear to justify the employment of the term "chromatin" for the element of the nucleus that stains with methyl green, the term "nuclein" having obtained a wider extension.

See also KOSSEL, in BEHRENS, KOSSEL, und SCHIEFFERDECKER's *Das Mikroskop*, &c., ii, p. 47; the same, in *Verh. d. physiol. Ges.*, Berlin, Oct. 21st, 1892; and MALFATTI, *Ber. d. naturw. med. Vereines in Innsbruck*, 1891–2.

For the microchemical detection of phosphorus in tissue-elements see the paper of LILIENFELD and MONTI, in *Zeit. f. physiol. Chemie*, xvii, 1892, p. 410 (Report in *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 332); also a short notice in HALLIBURTON's *Goulstonian Lectures*, 1893 (see *Brit. Med. Journ.*, March 11th, 1893, p. 505).

637. Cytological Fixing Agents.—The following is in great part taken from the numerous papers of FLEMMING in the *Arch. f. mik. Anat.* from the year 1879 onwards, and from his *Zellsubstanz, Kern- und Zelltheilung*.

Osmic acid ($\frac{1}{10}$ to 2 per cent.) preserves the form of the entire cell, but swells the nuclei and rounds off nucleoli. It renders the nuclear "reticulum" undiscernible. Picric acid, either concentrated or dilute, and chromic acid, 0·1 to 0·5 per cent., are to be preferred to alcohol and other agents for the study of the cells of *Vertebrates*. Shrinking and distortion of the nuclear figures (and, with picric acid, swellings of them) are to be expected, but other agents have the same defect to a much greater degree; alcohol especially causes *entanglement* of the filaments. Acetic acid does the same, and causes swelling besides. Stronger chromic acid solutions cause shrinking. Neither of these reagents is harmless as regards the nuclei of red blood-corpuscles. The salts of picric acid (potash-, soda-, and baryta-salts) are most harmful. Weak (*i. e.* not more than 1 per cent.) acetic, hydrochloric, or nitric acid, combined with clearing in glycerin and staining, may be

useful for bringing out reticula and nucleoli. Chloride of gold preserves the forms well, but generally leaves the nuclear structures unstained. Nitrate of silver is hopelessly uncontrollable in its action. Alcohol has much the effect of chromic acid, but often causes a much greater shrinking of the nuclei. Bichromate of potash and chromate of ammonia bring out very sharply the appearance of a reticulum, but these appearances cannot be accepted as true (l. c., p. 334, *et seq.*).

"*Those who seek to study cell-division by means of bichromate of potash or other chromic salts are hopelessly in the wrong road.*" And this because of the injurious action of the bichromate, *not* on the body of the cell, which it preserves well, but on the chromatin structures. Chromic salts are excellent reagents for general histological work, but not for nuclear structures. They dissolve nucleoli, destroy nuclear "networks," and swell up and distort karyokinetic figures to such a degree that the appearances obtained from them are merely unnatural caricatures of the true structure.

Altmann's nitric acid method is excellent for the purpose of hunting for cell-divisions in tissues; but the minute structure of the figures is not so well preserved as it is by means of chromic or picric acid. The same must be said of Kleinenberg's picro-sulphuric acid method. (I am not alone in holding that this is a most untrustworthy cytological reagent; see, for instance, Holl [*Sitzb. k. Acad. Wiss. Wien*, xcix, 1890, p. 311; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 89], who found that it frequently reduced chromosomes to the state of mere lumps, "Krümeln.")

There are two fixing mixtures which may be said to be classical for cytological studies, Flemming's **chromo-aceto-osmic acid** mixture, §§ 35, 36, and Hermann's **platino-aceto-osmic acid** mixture, § 51. As to the former of these, Flemming has the following explanations:—Attempts to omit the chromic acid from the formula did not give good results. The omission of acetic acid (as in Max Flesch's formula, § 34) causes the figures to be far less sharply brought out. The presence of acetic or formic acid in all osmium solutions is favorable to the precision of subsequent staining with hæmatoxylin, picrocarmine, or gentian-violet. But mixtures of osmic and acetic acid without chromic acid (Eimer) do not give such good results as the chromo-aceto-osmic acid mixture. Mixtures

of picric acid with osmic acid or with osmic and acetic acid (proportions of the latter as in the chromo-acetic-osmic mixture [§ 35], but of picric acid about 50 per cent.) fix quite as well as the chromic mixtures, but precise staining is even more difficult than with pure osmic acid preparations. Flemming concludes that the beneficial effects of the osmium in all these mixtures are to be ascribed to the instantaneous rapidity with which it kills, the function of the other acids of the mixture being to render the structures distinctly visible.

Mixtures containing osmic acid should therefore be employed whenever it is desired to fix the chromatic figures as faithfully as possible; whilst pure chromic acid should be taken whenever very sharp staining is the more important point.

For the study of the achromatic figures he recommends the chromo-acetic acid mixture (§ 31), followed by staining in hæmatoxylin (anilins do not give so good results for this purpose).

For the study of polar corpuscles he recommends the osmium mixtures, or pure chromic acid followed by staining with gentian-violet.

The above account stands nearly as it stood in the first edition. The state of things at present is as follows :—It is admitted by all competent observers that the chromo-aceto-osmic mixture is, with at most one or two possible exceptions, by far the best fixing agent for nuclei. But some observers have stated that it does not always preserve the cell-body well. This is a question that has been already discussed in §§ 35 and 36. I will only add here that after considerable experience I see no reason to distrust Flemming's mixture as a preservative of any kind of protoplasm, provided it be used in the proper way. It must be taken of the proper strength, it must be used with very small objects, so that it may act on all parts of them with its full strength, and not be filtered and diluted through thick walls of tissue before coming into contact with the object of study; and it must be allowed to act for the proper time.

This brings us to another point. There are *two* **chromo-aceto-osmic** mixtures—the old *weaker* one, and the new *stronger* one. Flemming recommended the strong one primarily as affording a means of differentiating kinetic chromatin from resting chromatin. He did not recommend it as a reagent for

general work. Whether of these two solutions should be used for general work? According to my experience, the strong solution *does* preserve both nuclear structures and caryoplasmic structures quite as faithfully at least as the old formula, and some structures most decidedly much better. Of course the one and the other should be taken according to the nature of the object you are dealing with; but I think it may safely be stated as a general rule that if you take the strong mixture, and fix thoroughly in it, you are not likely to go far wrong. And what is meant by a thorough fixation? Half an hour may be taken to be generally enough; but for very delicate things, such as the Nebenkern and the achromatic figure, at least eighteen hours ought to be given.

It only remains to point out that this doctrine is at variance with that expressed in the first edition and in the *Traité*, and with the earlier recommendations of Flemming; but I feel some confidence that it will not be called in question by the majority of workers at this subject. Of course it goes without saying that further precise evidence on the matter is very much to be desired.

As to the **platino-aceto-osmic** mixture of HERMANN, it has already been explained in § 51 that the point of superiority over Flemming's mixture that is claimed for it lies in a more faithful preservation of cytoplasm and achromatic structures. That the alleged superiority really exists appears to be the general opinion of those who have worked with this reagent. Flemming (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 685; *Zeit. f. wiss. Mik.*, viii, 3, p. 343) agrees that it gives a peculiarly sharp demonstration of spindle fibres, centrosomes, and polar corpuscles; but thinks that the chromo-aceto-osmic mixtures give a somewhat more faithful preservation of the chromatic elements.

Two or three of the fixing agents proposed by other writers may also rank as first-class reagents for this kind of work. There is RABL'S **chromo-formic** acid (§ 32). Fix in this for twelve to twenty-four hours, wash out well with water, and pass into alcohol. And there is the same observer's **platinum chloride** solution (§ 50). In Rabl's paper in *Anat. Anz.*, iv, 1889, p. 21, he recommends that *Salamandra* larvæ be fixed (for twenty-four hours) in a solution of from one tenth to one eighth per cent. strength. In his earlier work he used solu-

tions of 1—300 strength. Platinum chloride has the peculiarity of causing a slight shrinkage of the chromatin, which helps to bring into evidence the granules of Pfitzner and the longitudinal division of the chromosomes.

There remain to be mentioned several fixing agents with which very important work has been done. These, however, are, I think, not quite first-class reagents for the purpose, the brilliant results that have been obtained with them having been obtained rather in spite of their defects than on account of their good qualities. For instance, *acetic alcohol* is a reagent with which some of the most important work in recent cytology has been done—namely, much of that on the maturation and fecundation of the ovum of *Ascaris*. It is evident that for such an extraordinarily impenetrable object as the ovum of Ascaris, the employment of some such highly penetrating fluid as acetic acid is imperatively indicated, notwithstanding certain defects that it may have.

Carnoy (*La Cellule*, iii, 1, 1886, p. 6) used at first a mixture of three parts of absolute alcohol with 1 of glacial acetic acid ; later (*ibid.*, iii, 2, 1887) the chloroform mixture (§ 54). From five to fifteen minutes is enough for even the most resistent ova.

Van Beneden and Neyt (*Nouvelles Rech. sur la Féc. et la Division mitosique*, 1887) employed a mixture of equal parts of absolute alcohol and glacial acetic acid, or even pure acetic acid.

Acetic alcohol may be washed out with either pure alcohol, or with dilute glycerin (Calberla's formula would be a good one in many cases). For further details see *ante*, § 54.

M. Heidenhain (*Ueb. Kern u. Protoplasma*, 1892, p. 113) has been using corrosive sublimate, on account of its convenience, and, above all, on account of the great facility it affords for the employment of any kind of stain. The beautiful figures of attraction spheres and other cytoplasmic structures given in this paper show that the most brilliant results may be obtained by this means. But I would remark that the figures of nuclear structures appear to me less convincing. The author figures and describes under the name of "Lanthanin," an acidophilous caryoplasmic substance exhibiting a minutely reticular arrangement. The figures remind me of appearances which I frequently obtained in certain nuclei when working with sublimate some years ago, and which I regarded as artefacts, and in consequence was led to the abandonment of sublimate as a cytological fixative. I do not find in Heidenhain's paper that he has instituted control experiments to show that the reticular arrangement of his "lanthanin" is preformed in the nucleus, and would point out the need of such experiments before either the existence of the lanthanin reticulum or the fidelity of the reagent can be deemed established.

Altmann (*Arch. f. Anat. u. Entwickel.*, 1892, p. 223 ; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 331) has a new fixative for resting nuclei, viz. a 2·5 per cent. solution of ammonium molybdate to which is added about 0·25 per cent. of chromic acid. I have no personal knowledge or other information concerning this method.

Lemon juice (fresh, filtered) has been warmly recommended as a fixative for nuclei by VAN GEHUCHTEN (*Anat. Anzeig.*, iv, 1889, p. 52). Fix for five minutes, wash well with water and stain with methyl green, and examine in liquid of Ripart and Petit.

Heat has been recommended, but I believe it to be altogether objectionable. HENKING (*Zeit. f. wiss. Zool.*, xcix, 3, 1890, p. 503; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 211) has found that it totally destroys achromatic structures.

638. Chromatin Stains.—For fresh or lightly fixed tissues *methyl green* is the most generally useful nuclear stain. For the properties of this reagent see *ante*, § 109. It may be used either alone, or in the form of Ehrlich-Biondi mixture (see below).

Bismarck brown is another useful stain for such objects. It may be used in aqueous solution with acetic acid or with hydrate of chloral, or dissolved in dilute glycerin. Alum-carmine may occasionally be useful. Ehrlich's hæmatoxylin or Mayer's hæmalum will render services for osmium objects. Methyl violet, employed according to the method of GRASER (§ 111), may also be found a very useful stain.

For sections of hardened tissues we have the choice between the finer hæmatein stains (hæmalum, or Ehrlich's or Böhmer's hæmatoxylin) and those obtained by means of safranin, gentian violet, Victoriablau, and some other anilins, used according to the indirect or Flemming's method. This has been so fully explained in Chap. VIII that it is only necessary to refer the reader back to the paragraphs in question. Flemming's orange method has been given in § 258, and the Ehrlich-Biondi mixture in § 259, and will be further considered in the next section.

BABES'S supersaturated safranin stain (*Arch. f. mik. Anat.*, xxii, 1883, p. 361) may also occasionally be useful. It is as follows :—A supersaturated solution of safranin in water is warmed to 60° C. and filtered warm. On cooling, it becomes turbid through the formation of small crystals. Sections are placed in a watch-glass with some of this turbid solution, and the whole is warmed for a few seconds (till the liquid becomes clear) over a spirit-lamp. Allow the whole to remain for one minute, and wash out with water, and treat with alcohol and turpentine in the usual way. Tissues which do not take on the stain at once must be warmed over and over again. Clove oil must be avoided for clearing.

Renewed attention is called to OHLMACHER's observations on the employment of iodine or picric acid with safranin (§ 101).

M. HEIDENHAIN's iron hæmatoxylin stain has been described, § 197. It should be noted that the description of the results given by Heidenhain applies to preparations fixed in sublimate, and will presumably not apply to chromic sections.

639. Stains for Plasma and Achromatic Structures.—Foremost amongst these is the EHRLICH-BIONDI **mixture**, described in § 259. To the account there given there remain to be added some minutiæ regarding the application of the stain to cytological objects, particularly as regards the study of attraction-spheres and other achromatic elements. M. HEIDENHAIN (*Über Kern und Protoplasma*, Engelmann, 1892, p. 116; see also *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202, a very full report) gives the following instructions. Preparations made with the usual mixture are liable to fade; by acidifying the mixture a stronger and more sharply selective stain is obtained, which does not fade. But too much acid must not be added, as this would cause a staining of the interfilar substances. Heidenhain's directions are as follows:

Take some of the "commercial stock solution" (by which appears to be meant the mixture as given in § 259), and dilute it with distilled water in the proportion of about 6 parts of the mixture to 400 of water. Fill two beakers with distilled water, and add to each a few drops of the diluted solution. The coloured liquid thus obtained will show, at the same time, a red tint owing to the rubin, a yellow tone owing to the orange, and a greyish tone derived from the methyl green. Now add to one of the beakers, with continual agitation, drop by drop, a 1 : 500 solution of acetic acid until the colour of the liquid turns to a strong crimson, the former yellow tone disappearing, and the grey of the methyl green becoming less marked. The two beakers serve as standards for the degree of acidity that should be given to the staining bath. The dilute solution that you made by diluting the original stock solution* is now acidified with the dilute acetic acid, added drop by drop with continual agitation, and from time to time

* "Das aus der Stammlösung durch Verdünnung gewonnene Quantum." The passage is somewhat misty.

a few drops are taken from it and added to a beaker of distilled water until there is obtained the crimson tint of the first test-beaker. Addition of acid should then cease, and the staining bath is ready. If the preparations should not turn out quite satisfactory a small further quantity of acid may be added.

Before staining, sections should be treated for a couple of hours with 0·1 per cent. acetic acid, then for ten to fifteen minutes with officinal tincture of iodine, and be rinsed with alcohol before bringing into the stain, in which they should remain for twelve to eighteen hours. The treatment with acid is necessary in order to ensure having the sections *acid* on mounting in balsam. The primary object of the iodine is to remove any sublimate from the preparations (Heidenhain's descriptions refer to sublimate objects, but Ehrlich-Biondi mixture will work with chrome objects); but it also enhances the power of staining of the chromatin with methyl green, and produces a more selective staining of protoplasmic elements.

Instead of acidifying with acetic acid, the mixture may be modified by increasing the proportion of acid fuchsin, a suggestion which is, I gather, also due to Heidenhain (I do not know whether it has been published). Dr. Grübler has sent me a mixture of the three colours in the dry state in such proportions that you have only to dissolve 0·4 grm. of the mixture in 100 c.c. of water, and add 7 c.c. of a 0·5 per cent. solution of Säurefuchsin. I greatly regret that I am unable to make trial of this preparation before going to press.

Benda (*Verh. physiol. Ges. zu Berlin*, Dec. 18th, 1891, Nos. 4 u. 5; see also *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 516) proceeded as follows (testes of Mammalia) :—Sections stained for twenty-four hours in anilin-water safranin solution, then for about half a minute in a solution of 0·5 grm. "Lichtgrün F. S." or Säureviolett (Grübler) in 200 c.c. of alcohol, dehydrated, and mounted in balsam. Chromatin red, archiplasma bright green (or violet), centrosomata and "Spitzenknopf" (of spermatozoa) sometimes green, sometimes red. The chromatoid "Nebenkörper" of Hermann stains red.

The achromatic structures of the ova of *Ascaris* are best demonstrated, according to van Beneden and Neyt (*Nouv. Rech. sur la Fécond. et la Div. mitosique*), by fixing with acetic

alcohol (*ante*, § 637), and bringing the ova into one-third glycerin in which is dissolved a little malachite green. The "sphères attractives" stain green. See also BOVERI's *Zellen-Studien* (in *Jen. Zeitschr. f. Naturw.*, xxi, 1887, p. 423, or sold separately).

The osmium and pyroligneous acid method of HERMANN has been described in § 233. It is said to give a so abundant reduction of the osmium that no other stain is necessary. In the same place HERMANN also recommends a modification of the hæmatoxylin impregnation method of PAL (§ 692):—Testes of *Proteus* put for twelve to eighteen hours into hæmatoxylin, 1 grm.; absolute alcohol, 70 c.c.; water, 30 c.c., in the dark, and washed out for the same time in 70 per cent. alcohol, also in the dark. Sections having been made are treated with dilute pale rose-coloured solution of permanganate of potash till they turn ochre-coloured, rinsed in water, and washed out for removal of the brown peroxide of manganese in Pal's oxalic acid mixture diluted with 5 to 10 volumes of water. They are finally stained for three to five minutes in safranin.

The iron-hæmatoxylin stain of BÜTSCHLI and that of M. HEIDENHAIN have been given in §§ 196, 197.

For BENDA's copper-hæmatoxylin, see *Arch. f. mik. Anat.*, xxx, p. 49; also *Journ. Roy. Mic. Soc.*, 1888, p. 158, or *Zeit. f. wiss. Mik.*, v, 1888, p. 499.

FLEMMING's orange method has been given § 258.

HENNEGUY's permanganate method has been given § 106.

RABL's method (platinum chloride, hæmatoxylin, safranin).—See *Morph. Jahrb.*, x, 1884, p. 215.

PLATNER's.—See *Arch. f. mik. Anat.*, xxxiii, 1889, p. 125. His Kernschwarz has been discussed in § 208.

640. Nucleus of BALBIANI ("Noyau Vitellin," "Cellule Embryogene") (*Zool. Anz.*, 1883, p. 659).—This may be observed in the fresh state, without the addition of any reagent, in the ova of some animals, amongst others a great number of Arachnida and Myriapoda. It may be brought out more distinctly by treating the ova with a mixture of equal parts of acetic acid and 1 per cent. osmic acid, to which is added a little sodium chloride. This mixture does not render ova so granular as pure dilute acetic acid.

641. Cell-granules.—These for the most part undoubtedly metabolic products are best studied in gland-cells and blood- and lymph-corpuscles, and in certain elements belonging to the group of connective tissues, and the reader is therefore referred to Chap. XXXI for the appropriate methods.

For ALTMANN's "Bioblasts," see, besides that author's

Studien über die Zelle, 1886, his *Die Elementarorganismen,* Leipzig, 1890, and a paper in *Arch. f. Anat. u. Entwickel.,* 1892, p. 223; also *Zeit. f. wiss. Mik.,* vii, 2, 1890, p. 199; ix, 3, 1893, p. 331; and L. and R. Zoja, in *Mem. R. Ist. Lombardo di Sci. e Lettere,* xvi, 3, vii, p. 237. Omitting minutiæ and variations, it may be said that these granules may be demonstrated by fixing for twenty-four hours in a mixture of equal parts of 5 per cent. bichromate of potash and 2 per cent. osmic acid, imbedding in paraffin, staining sections for a minute on the slide held over a flame with a solution of 20 grms. of acid fuchsin in 100 c.c. of anilin water (§ 101), and washing out with saturated alcoholic solution of picric acid diluted with 2 volumes of water, heat being used as before to aid the differentiation, and finally clearing with xylol and mounting in balsam.

642. Mounting.—For fresh objects you have so large a choice of mounting media that you may take whatever liquid gives you the best optical results. Sections of hardened tissue should always (if possible) be mounted in damar (or colophonium), not balsam, as the slightly lower index of damar or colophonium solutions gives more powerful images of very delicate details. If you have to deal with objects so delicate that you have cause to fear mechanical injury to them on putting them into damar, they may be mounted in thickened turpentine (or cedar oil, but turpentine is preferable on account of its lower index). Rabl has lately been using methyl alcohol as an examination medium for dehydrated objects; but the preparations do not keep in this. Castor oil may be tried (Grenacher). I have not had good results with it.

643. Other General Cytological Methods.

Strasburger's **Methods** (see *Arch. f. mik. Anat.,* xxi, 1881, p. 477).

Uskoff's **Nitric Acid Method** (see *Arch. f. mik. Anat.,* xxi, 1882, p. 292).

Pfitzner's **Sulphate of Soda Method** (see *Morphol. Jahrb.,* xi, 1885, p. 54).

Tizzoni (*Bull. delle Sci. Med. di Bologna,* 1884, p. 259).

Baumgarten (*Zeit. f. wiss. Mik.,* i, 1884, p. 415).

Bizzozero (*ibid.,* iii, 1886, p. 24).

Babes (*ibid.,* iv, 4, 1887, p. 470).

Zwaardemaker (*ibid.,* iv, 2, 1887, p. 212).

Nissen (*Arch. f. mik. Anat.,* 1886, p. 338) stains material fixed in Flemming and sectioned, by the method of Gram (see above, § 102).

SCHOTTLÄNDER (*Arch. f. mik. Anat.*, xxxi, 1888, p. 426; *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 515—cell-division in the corneal endothelium).

644. Cytology of the Ovum (*Ascaris*).

VAN BENEDEN (*Arch. de Biol.*, iv, 1883, p. 279).

CARNOY (*La Cellule*, ii, 1, 1886, pp. 17, 18; iii, 1, 1886, p. 6; iii, 2, 1887).

VAN BENEDEN et NEYT (*Nouv. Rech. sur la Féc.*, &c., separate, 1887, or *Bull. Acad. roy. d. Sc. de Bruxelles*, 1887, iii sér., xiv; also *Journ. Roy. Mic. Soc.*, 1888, p. 508).

BOVERI (*Zellen-Studien*, in *Jen. Zeit. f. Naturw.*, xxi, 1887, p. 423, or separate; also *Journ. Roy. Mic. Soc.*, 1888, p. 664).

ZACHARIAS (*Anat. Anz.*, iii, 1888, p. 24; also *Journ. Roy. Mic. Soc.*, 1888, p. 663).

VAN GEHUCHTEN (*ibid.*, p. 237).

KULTSCHITZKY (*Arch. f. mik. Anat.*, xxxi, 1888, p. 567) recommends for fixing a mixture of 3 parts of acetic ether with 1 part of absolute alcohol. Clears for mounting in concentrated acetic acid, and mounts in a mixture of acetic acid and balsam. An excellent *résumé* of the technical part of these papers is to be found in *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 367.

Other Ova.

HERTWIG, O. (*Morph. Jahrb.*, x, 1884, p. 338, *Rana*).

HERTWIG, O. and R. (*Jen. Zeit. f. Naturw.*, xix, 1885, p. 124).—Fecundation of *Strongylocentrotus* (Echinodermata). Careful study of cell-anæsthetics. Abstracts in *Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 505, and *Journ. Roy. Mic. Soc.*, 1887, p. 835.

BOVERI (*Zellen-Studien*, iii [Echinodermata], in *Jen. Zeit. f. Naturw.*, xxiv, 1890, p. 314, or separate).

645. Spermatological Methods.—One of the great difficulties here met with consists in getting a sufficiently rapid fixation of spermatids and spermatozoa, some of which are probably the most rapidly contracting elements that exist. For fresh material, teased on the slide, strong solution of permanganate of potash is very useful, being the most rapidly fixing agent that I know of. Tincture of iodine is also useful. For staining, gentian violet or dahlia should be used as far as possible, as they have a special affinity for spermatic cells. For mounting aqueous preparations, solution of Ripart and Petit, or some such mercurial fluid as those of Pacini, are indicated.

As to the Nebenkern, which is here so important an element, see *ante*, § 639.

Besides the papers quoted for cytological methods, and besides the numerous publications of V. LA VALETTE ST. GEORGE, in *Arch. f. mik. Anat.*, see the following:

CARNOY (*La Cellule*, i, 1885, p. 209, Arthropoda).

GILSON (*ibid.*, i, 1885, pp. 40, 56, 58, 87, 121, 141, and ii, 1886).—Here is a useful fixing medium for fresh teased preparations—*iodised osmic acid*

made by adding a little of a concentrated solution of iodine in iodide of potassium to 2 per cent. osmic acid; and some other formulæ useful for their special objects (most various forms of Arthropoda).

Swaen et Masquelin (*Arch. de Biol.*, iv, p. 752).—Selacians, *Salamandra, Bos.*

Flemming (*Arch. f. mik. Anat.*, xxix, 1887, p. 387; abstracts in *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 236, and *Journ. Roy. Mic. Soc.*, 1888, p. 146).

Prenant (*Intern. Monatschr. f. Anat.*, iv, 1887, p. 358; abstracts in *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 84; and *Journ. Roy. Mic. Soc.*, 1888, p. 844 [Mammalia]).

Renson (*Arch. de Biol.*, iii, 1882, p. 302).

Benda (*Arch. f. Anat. u. Phys.; Phys. Abth.*, 1886, p. 186).—For his modification of Weigert's hæmatoxylin stain, see below.

Jenson (*Arch. de Biol.*, iv, 1883, p. 11, *et passim* [Selacians and Invertebrates]).—Concentrated solution of oxalic acid for hardening.

Pictet (*Mitth. a. d. zool. Stat. zu Neapel*, Bd. x, Hft. 1, 1891, p. 89).—Instructive series of figures showing the very different forms assumed by spermatozoa of *Strongylocentrotus* under treatment by twenty-three different reagents. The most faithfully-preserving examination fluid was found to be a 5 to 10 per cent. solution of *chloride of manganese* containing a small quantity of dahlia dissolved in sea water.

For some reactions of divers stains on the nucleus and Nebenkern of spermatozoa, see Leclercq, *Bull. Acad. Roy. Belg.*, lx, 1890, p. 138; *Journ. Roy. Mic. Soc.*, 1890, p. 816.

Balbiani (*Leç. sur la Génér. des Vertébrés*, 1879, p. 244).—Double-stain sections with picro-carmine and methyl green. The latter stages of differentiating cells stain blue, less advanced ones lilac; and the cells which form the walls of the tubes stain only red.

Ryder, in Whitman's *Meth. of Research*, p. 52.—Sections of hermaphrodite gland of Lamellibranchiata stained for two or three hours in a mixture of equal parts of concentrated alcoholic solution of safranin and methyl green, diluted with 8 volumes of water, washed out for five to ten minutes in alcohol, and mounted in balsam. Nuclei of ova, red; heads of spermatozoa, bluish green.

If sections of a gland of *Helix* be stained with methyl green and eosin, the ova are stained rose colour, and the spermatozoa bluish green (Henneguy, in *Traité des Meth. Techn.*, Lee et Henneguy, p. 343).

Auerbach (*Sitzb. k. Acad. Wiss. Berlin*, xxxv, 1891, p. 713; see *Journ. Roy. Mic. Soc.*, 1891, p. 714; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 81) has made some observations which seem to point to a qualitative chemical difference in the composition of the nuclei of male and female sexual products. Sections of male and female sexual products, prepared in precisely the same way, were fixed together on slides and stained with pairs of the following dyes (used either mixed or in succession):

Blues.	*Reds.*
Methyl green.	Carmine.
Emerald green.	Eosin.
Victoria blue.	"Echtroth."
Methylen blue.	Fuchsin.
(Sometimes) Hæmatoxylin.	Orange.
	Orange with fuchsin.
	Rosanilin sulphate.

It was found that the chromatin (and also the nucleoli) of the female elements constantly took the red stain, whilst that of the male elements constantly took the blue stain. Auerbach constantly arrives at the generalisation that female chromatin is erythrophilous, male chromatin cyanophilous.

His procedure for staining was to make aqueous solutions of the two colours of approximately equal intensity of tint, and either to stain in them successively or to mix equal volumes of them for a staining bath.

The subject appears to call for further research.

CHAPTER XXVII.

TEGUMENTARY ORGANS.

646. Epithelium.—One of the chief methods of obtaining preparations giving instructive surface views of epithelia is the nitrate of silver method. For this see *ante*, § 212, *et seq.*, in the chapter on "Impregnation Methods." The reader may also consult with advantage the admirable instructions given by RANVIER in his *Traité technique*, p. 246, *et seq.*, and the memoir of TOURNEUX and HERMANN in the *Journ. de l'Anat.*, 1876, p. 200.

The perchloride of iron and pyrogallic acid method of the HOGGANS, § 231, and the osmic acid and pyrogallic acid process, § 233, may also be found useful here. But in many cases impregnation with methylen blue will doubtless be found preferable (see § 113, *et seq.*, and especially §§ 120 and 121).

KROMAYER'S process for demonstrating his intra- and intercellular fibrils of epithelia (*Arch. f. mik. Anat.*, xxxix, 1892, p. 141; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 84, and ix, 3, 1893, p. 355) is as follows:—Sections are stained for five minutes in a mixture of equal volumes of anilin water (§ 101) and concentrated aqueous solution of methyl violet 6 B. They are well washed in water, and treated with solution of iodine in iodide of potassium until they become blue-black (one to thirty seconds). They are again washed with water, dried with blotting-paper, and treated with a mixture of 1 vol. of anilin to 2 vols. of xylol until sufficiently differentiated, when they are brought into pure xylol. Very thin sections will require more xylol in proportion to the anilin, viz. 1 : 3 or 1 : 4; thicker ones may require more anilin, viz. 3 : 5 or 3 : 3. Gentian or Krystallviolett will do instead of methyl violet, but not quite so well. For a discussion of the points of difference between the fibrils of Kromayer and the fibres of HERXHEIMER, see EHRMANN, and JADASSOHN, *Arch. f. Der-*

matol. u. Syphilis, 1892, 1, p. 303; *Zeit. f. wiss Mik.,* ix, 1893, p. 356.

Sections are easily made by the usual methods. The best hardening agent for *skin* appears to be Müller's solution. This was the conclusion of F. E. Schultze in 1867 (*Arch. f. mik. Anat.,* p. 145), and it is that of Tizzoni, the author of important researches on this organ (*Bull. delle Sc. med. di Bologna,* 1884, p. 259), and of Behn (*Arch. f. mik. Anat.,* xxxix, 1892, p. 581). Simple bichromate of potash solution will do about as well.

For *glandular epithelium* it is frequently better to employ a chromic acid liquid, or osmic acid (see, for example, Ranvier, loc. cit., p. 258, *et seq.*), or absolute alcohol (Blaue, *Arch. f. Anat. u. Phys.,* 1884, p. 231); "Kleinenberg" is not so good.

Prickle-cells and Intercellular Canals.—Besides maceration, which is one of the most important of the methods for the study of these objects, impregnation may be useful. Mitrophanow (*Zeit. f. wiss. Zool.,* 1884, p. 302, and *Arch. f. Anat. u. Phys.,* 1884, p. 191) recommends the following process:—Wash with distilled water the tail of an axolotl larva; put it for an hour into 0·25 per cent. solution of gold chloride with one drop of hydrochloric acid to a watch-glassful of the solution; wash, and reduce in a mixture of one part of formic acid with six parts of water.

Macerating Media.—For soft epithelia, mild macerating agents, such as iodised serum, one-third alcohol, saliva, or Schultze's mixture of saliva and solution of Müller, or a mixture of saliva with three to four volumes of physiological salt solution (Bizzozero, *Intern. Monatschr. f. Anat.,* 1885, p. 278);—for hard epithelia, energetic dissociating agents, such as 40 per cent. solution of caustic potash.

Minot (*Amer. Natural.,* xx, 1886, p. 575; cf. *Journ. Roy. Mic. Soc.,* 1886, p. 872) recommends maceration for several days in 0·6 per cent. solution of sodium chloride containing 0·1 per cent. of thymol, which allows the isolation of the epidermis of embryos, and is useful for the study of the development of hairs.

Another method, given by Mitrophanow (see *Zeit. f. wiss. Mik.,* v, 4, 1888, p. 513), is as follows:—An embryo of axolotl is fixed for a quarter of an hour in 3 per cent. nitric acid, and then brought into one-third alcohol. After an hour the

epidermis begins to come away in places; and if the embryo be put for twenty-four hours into stronger spirit, it will come away almost entirely.

For the purpose of separating the epidermis from the corium, LOEWY (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 159; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 222) recommends macerating for twenty-four to forty-eight hours, at a temperature of about 40° C., in 6 per cent. pyroligneous acid. Acetic acid of $\frac{1}{3}$ per cent. (PHILIPPSON) is also good.

The elements of *hairs* and *nails* may be isolated by prolonged maceration in 40 per cent. potash solution, or by heating with concentrated sulphuric acid.

Horny tissues stain well in safranin or gentian violet (REINKE, *Arch. f. mik. Anat.*, xxx, 1887, p. 183; *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 383).

647. Tactile Hairs.—RANVIER (*Traité*, p. 914) recommends for the study of the nerve-endings the boiled formic acid and gold-chloride method, § 225. A tactile hair having been isolated with its bulb, and its capsule incised, is put for about an hour into the formic acid and gold-chloride mixture, the gold is reduced in slightly acidulated water, hardening is completed in alcohol, and longitudinal and transverse sections are made. For DRASCH's method see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492.

648. Skin Nerves.—WOLTERS (*Arch. f. Dermatol. u. Syphilis*, 1892; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 360) employs the hæmatoxylin method, § 726, *post*.

649. Intra-epidermic Nerve-fibres.—May be studied by the gold method. RANVIER (*Traité*, p. 900) recommends the boiled formic acid and gold-chloride method, § 225.

He also (p. 910) recommends this method for the study of the tactile menisci of the pig's or mole's snout.

Pieces of skin are impregnated as directed § 225, and after reduction are brought into alcohol, which completes the hardening, and stays the further reduction of the gold. Sections are then made.

For the study of the tactile menisci of the snout, Ranvier also recommends the lemon juice and gold-chloride method, § 226.

The methylen blue impregnation method, § 113, *et seqq.*, should also be employed in the study of these nerve-endings.

650. Innervation of the Muzzle of the Ox (Cybulsky, *Zeit. f. wiss. Zool.*, 1883, p. 635; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 555).—Cybulsky employs the method of Hénocque given above, § 228.

651. Tactile Corpuscles (Fischer, *Arch. f. mik. Anat.*, 1875, p. 366).—Fischer employed the gold method of Löwit—see § 224. Ranvier (*Traité*, p. 918) also recommends this method, as well as his two gold methods, §§ 225, 226. Pieces of skin are first impregnated whole, then hardened by alcohol and sectioned. He finds (as do other authors) that osmic acid and picro-carmine are invaluable aids to the study of these structures, and to that of the corpuscles of Pacini. Purpurin and hæmatoxylin may also be used for after-staining. See Ranvier, *Traité*, p. 919; and see also Langerhans, *Arch. f. mik. Anat.*, 1873, p. 730; and Kultschizky, *ibid.*, 1884, p. 358.

652. Corpuscles of Herbst and Corpuscles of Grandry (Carrière, *Arch. f. mik. Anat.*, 1882, p. 146).—Take fresh beaks of ducks, remove the skin and papillæ from the margins, and put pieces into formic acid (50 per cent.) for twenty minutes or until transparency is attained; remove the corneous layer of epithelium; rinse in water; gold chloride 1 per cent. (twenty minutes); rinse in water; Pritchard's solution (amyl-alcohol 1 per cent., formic acid 1 per cent., water 98 per cent.) from midday till next morning (in the dark); rinse in water; treat with alcohol, imbed in paraffin, and make sections.

It is important to take only *small quantities* of gold chloride, not more than about 10 c.c. of the solution to "quite a number" of pieces of skin and papillæ. On the other hand, *large* quantities of Pritchard's solution should be employed.

Dogiel (*Arch. f. Anat. u. Entwickel.*, 1891, p. 182; *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 520) prefers the methylen blue method (Chap. IX). Four per cent. solution of methylen blue, warmed to 40° C., is injected into blood-vessels of the heads of ducks or geese; pieces of skin are removed from the beaks, sectioned in pith, and the sections brought on to slides

and moistened with aqueous or vitreous humour from the animal, and left for a few minutes exposed to the air (it is well to add to the aqueous or vitreous humour a few drops of $\frac{1}{16}$ per cent. methyl blue solution). After about ten to thirty minutes the nerve-endings are seen to be stained, and the sections are then brought into picrate of ammonia, and treated as described in § 119.

653. Corpuscles of Krause (Cornea and Conjunctiva bulbi) (Dogiel, *Arch. f. mik. Anat.*, xxxvii, 1891, p. 602; *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 519).—A fresh bulb should be enucleated *in toto*, and incised along a line running parallel to the equator and 5 to 8 mm. behind the corneal margin. The anterior segment thus obtained is freed from the ciliary body, lens, &c., the conjunctiva being left *in situ* on the cornea. It is then cut into pieces which are stained for an hour to an hour and a half in methylen blue + aqueous humour, as described in § 117, and further treated for examination and preservation as described in § 119.

See also Longworth's methods, *Arch. f. mik. Anat.*, 1875, p. 655.

654. Papillæ Foliatæ of the Rabbit (Hermann).—See *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 524.

655. Olfactive Organs of Vertebrates (Dogiel, *Arch. f. mik. Anat.*, 1887, p. 74).

656. Organs of a "Sixth Sense" in Amphibia (Mitrophanow).—See *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 513. This paper contains some details as to staining with "Wasserblau," for which see also *Biol. Centralb.*, vii, 1887, p. 175.

657. Nerve-endings in Tongue of Frog (Fajerstajn [Feuerstein], *Arch. de Zool. expér. et gén.*, vii, 1889, p. 705; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 357).—Amongst other methods for the study of the terminal discs, the methylen blue method is recommended. Solution of 1 part methylen blue to 800 of 0·6 per cent. salt solution should be injected through the abdominal vein (not through the vena cutanea magna), the animal being curarised or etherised. After injecting, care must be taken that air has access to the mouth. Injection through the dorsal lymph-sac will also succeed, and simple

pouring of the stain into the mouth sometimes gives good results. See also Chap. IX.

658. Cornea.—There are three chief methods for the study of the corneal tissue—the methylen blue method, the silver method, and the gold method.

For the *methylen blue method* see Chap. IX, particularly §§ 120 and 121.

Negative images of the corneal cells are easily obtained by the dry *silver method* (Klein). The conjunctival epithelium should be removed by brushing from a living cornea, and well rubbing the corneal surface with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (Ranvier) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation, by which the cells are brought out with admirable precision.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating a negatively impregnated cornea with weak salt solution or weak solution of hydrochloric acid (His).

But the best positive images are those furnished by *gold chloride*. Ranvier prefers his lemon-juice method to all others for this purpose (see § 226). It is important that the cornea should *not remain too long in the gold solution*, or the nerves alone will be well impregnated.

Ranvier also recommends this method as being the best for the study of the nerves.

Rollett (Stricker's *Handbuch*, p. 1115) recommends a double impregnation with silver followed by gold for obtaining gold-stained *negative* images. A cornea having been treated *for a short time only* with 0·5 per cent. silver nitrate solution, and the silver reduced, is treated with 0·5 per cent. gold-chloride solution. The brown stain of the silver disappears immediately the preparation is placed in the gold solution; after a few minutes the preparation is exposed to the light in acidulated water. Reduction of the gold rapidly takes place, and in the place of the former brown stain of the silver the

ground-substance shows the well-known blue of reduced gold. The cells are, however, visible, being recognisable by their granular appearance and pale yellow tint.

RENAUT (*Comptes Rend.*, 1880, 1er sem., p. 137) gives the following process for corneal corpuscles :—Cornea of frog: formic acid, 20 per cent., ten minutes; gold chloride, 1 per cent., twenty-four hours; formic acid, 33·3 per cent., twenty-four hours.

659. Cornea, other Methods (ROLLETT, Stricker's *Handb.*, p. 1102).—Rollett strongly recommends the following plan :—A fresh cornea is placed (in humor aqueus) in a moist chamber, and exposed to the action of iodine vapour. As soon as it has become brown the epithelium may easily be peeled off. If the reaction is not complete the cornea may be put back into the iodine chamber. When sufficient iodine has been absorbed the preparation may be examined, and it will be found that the network of corneal cells is brought out with an evidence hardly inferior to that of gold preparations. The method never fails, which is not the case with the gold method. It is admirable as a fixing method.

For dissociation of the fibres Rollett recommends maceration in a solution of permanganate of potash or a mixture of this solution with alum. As soon as the tissue has become brown it is shaken in a test-tube with water, and breaks up into fibres and bundles of fibres.

TARTUFERI (*Anat. Anz.*, v, 1890, p. 524; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 365) has the following method for demonstrating corneal cells and the ramifications of their processes :—A cornea is placed for three or more days in a solution of 15 grammes of hyposulphite of soda to 100 c.c. of distilled water, kept at a temperature of about 26°, then removed for two days into water containing very finely powdered chloride of silver (if left longer the corneal cells will not be stained, but innumerable elastic fibres will be demonstrated). The preparations are said to be permanent.

For cell-division in the membrane of Descemet, see SCHOTTLÄNDER'S methods, *ante*, § 643.

660. Crystalline (Hardening of) (LÖWE, *Arch. f. mik. Anat.*, 1878, p. 557).—A fresh bulb is placed in a vessel containing

several litres of 1 per cent. bichromate of potash solution, which is frequently changed for stronger solutions until the strength of a cold saturated solution is attained. The bulb must remain in this for at least *a year and a half*, in order that the crystalline may attain the right degree of hardness. For **Maceration**, use Max Schultze's sulphuric acid solution, *supra*, § 546.

CHAPTER XXVIII.

MUSCLE AND TENDON (NERVE-ENDINGS).

Striated Muscle.

661. Sections (ROLLETT, *Denkschr. math. naturw. Kl. k. Acad. Wiss. Wien*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 92).—Besides the usual section methods, the following methods of Rollett should be noted:—(1) The method mentioned above, § 321, of freezing living tissue in white of egg. (2) The same method applied to recently fixed muscle. (3) For hardened muscle, Rollett prefers celloidin. He stains for several hours in Renaut's hæmatoxylic glycerin diluted with water to a very light violet tint. Dehydrate with alcohol, clear with origanum oil, and mount in dammar.

662. Dissociation.—See Chap. XXIII.

LANGERHANS' methods for *Amphioxus* (*Arch. f. mik. Anat.*, 1875, p. 291).—For isolation of the muscle-plates macerate the fresh animal in 20 per cent. nitric acid.

For isolation of the nervous system macerate an animal for three days in 20 per cent. nitric acid, then place it for twenty-four hours in water, and shake forcibly. The *whole* of the nervous system may thus be separated, almost down to the finest peripheral terminations of nerves.

663. Muscle-cells.—For the study of these and allied subjects see, *inter alia*, BEHRENS, KOSSEL, und SCHIEFFERDECKER, *Das Mikroskop*, &c., vol. ii, pp. 154—161; also, for the application of the gold method to the study of muscle-cells, SCHÄFER, *Proc. Roy. Soc.*, xlix, 1891, p. 280; or *Journ. Roy. Mic. Soc.*, 1891, p. 683.

664. Sarcolemma.—Besides the places quoted in last section, see SOLGER, *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 189 (small pieces of fresh muscle teased and examined in cold saturated solution of carbonate of ammonia).

665. Nerve-endings.—For the study of nerve-endings in muscle, both motor and sensory, the four chief methods are the methylen-blue method, the gold method, the silver method, and the bichromate of silver method of Golgi.

666. Nerve-endings—the Methylen-blue Method.—The principles of the impregnation of nerve-tissue with methylen blue have been explained in Chap. IX.

Biedermann's procedure for the muscles of *Astacus* has been indicated in § 117 (see also *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 65). After impregnating as there directed the carapace should be opened, and the muscles exposed to the air in a roomy moist chamber for from two to six hours, in order that the stain may differentiate. The abdominal and caudal muscles are those which give the best results.

For *Hydrophilus piceus,* Biedermann proceeded by injecting 0·5 c.c. of methylen-blue solution between the ultimate and penultimate abdominal rings, in the ventral furrow, and keeping the animals alive in water for three to four hours. After this time the thorax should be opened by two lateral incisions, and the muscles of the first pair of legs (which are the most suitable) removed and exposed to the air for three or four hours in a moist chamber, and finally examined in salt solution.

Gerlach (*Sitzb. k. math.-phys. Cl. k. bayer. Akad. Wiss. München,* 1889, ii, p. 125; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 220) injected frogs, either through the abdominal vein or through the aorta, with 4 to 5 c.c. of a 1 : 400 solution in 1 per cent. salt solution, and examined pieces of muscle (preferably the head and eye muscles) in serum of the animal, afterwards fixing the preparations with picrate of ammonia and mounting in glycerin jelly.

The procedure of Dogiel has been given in § 117.

667. Nerve-endings—the Gold Method.—Fischer (*Arch. f. mik. Anat.*, 1876, p. 365) used the gold method proposed by Löwit (*Wien. Sitzgsber.*, Bd. lxxi, Abth. 3, 1875, p. 1), and employed by himself in his researches on the tactile corpuscles (*Arch. f. mik. Anat.*, xii, p. 366). See *ante,* § 224.

Biedermann, in the paper quoted in the last section, recommends for *Astacus* a similar procedure, the preliminary treat-

ment with formic acid being omitted, and the muscles being put for a couple of days into glycerin after reduction in the acid.

The procedure of TRINCHESE (*Mem. R. Accad. Ist. Bologna*, 5, ii, p. 279; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 238) is also practically identical.

RANVIER (*Traité*, p. 813) finds that for the study of the motor terminations of Batrachia the best method is his lemon-juice and gold-chloride process (§ 226). The delicate elements of the arborescence of Kühne are better preserved by this method than by the simple method of Löwit.

For the study of the motor plates of reptiles, fishes, birds, and mammals, he finds (*ibid.*, p. 826) that his formic acid and gold-chloride method, § 225, gives preparations infinitely superior to those obtainable by the method of Löwit; but the lemon-juice method is still better, especially for lizards and mammals. The branches of the terminal arborescence are more regular than in preparations obtained by the formic acid process.

668. Nerve-endings—the Silver Method.—RANVIER finds that the silver nitrate method of Cohnheim is also useful. He employs it as follows (*ibid.*, p. 810) :—Portions of muscle (gastrocnemius of frog) having been very carefully teased out in fresh serum are treated for ten to twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swelled up to their normal dimensions (the swelling induced by the acid serving to make up for the shrinkage caused by the nitrate of silver). They are then examined in a mixture of equal parts of glycerin and water.

This process gives *negative* images, the muscular substance is stained brown, except in the parts where it is protected by the nervous arborescence, which itself remains unstained. The gold process gives *positive* images, the nervous structures being stained dark violet.

669. Nerve-endings—the Bichromate of Silver Method.—The osmium bichromate and silver method of Golgi has been successfully applied by RAMÓN Y CAJAL to the study of the terminations of nerves and of tracheæ in the muscles of insects,

and is doubtless susceptible of still wider applications. The process used by him is given below, § 714.

670. Nerve-endings—other Methods.

Bremer (*Arch. f. mik. Anat.*, 1882, p. 195).—Impregnate according to the method of Fischer, *ante*, § 224, and soak for two or three weeks in glycerin containing 20 per cent. of formic acid. Mount in glycerin containing 1 per cent. of formic acid.

Ciaccio (*Journ. de Micrographie*, 1883, p. 38).—Lemon juice followed by 1 per cent. solution of double chloride of gold and cadmium, and reduction partly in the light and partly in concentrated formic acid in the dark.

Wolff (*Arch. f. mik. Anat.*, 1881, p. 355).—Study of the living muscle in salt solution.

Carl Sachs (*ibid.*).—Dilute acetic acid followed by very weak picric acid for twenty-four hours. After mounting in glycerin, this gives very transparent preparations.

Krause (*Intern. Monatschr. f. Anat. u. Hist.; Zeit. f. wiss. Mik.*, 1885, p. 547).—A muscle is put for three or four hours into concentrated solution of oxalic acid, then boiled for two minutes in water, treated for twenty-four hours with 0·1 per cent. osmic acid, and mounted in glycerin.

Negro (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 240).—Make the following solution:

Concentrated solution of ammonia alum . .	180 parts.
Grübler's saturated alcoholic solution of hæmatoxylin	2 „

Mix, and let the mixture stand for a week exposed to the air, and add 25 c.c. each of methyl alcohol and glycerin. A drop of this is brought on to a fresh preparation of muscle of *Tropidonotus natrix*, *Lacerta viridis*, or *Rana*, and in a few minutes the stain is washed out and the preparation mounted in balsam.

Boccardi (see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492).—Preparations treated according to Ranvier's lemon-juice method or his formic acid method are washed in distilled water, and put for a couple of hours into oxalic acid of from 0·1 to 0·25 or 0·30 per cent., or, better, into a mixture of—

Pure formic acid	5 c.c.
1 per cent. oxalic acid	1 „
Distilled water	25 „

Then washed and mounted in glycerin.

Kühne (*Zeit. f. Biol.*, xxiii, v, 1887, p. 1; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 495).—This paper contains a critical review of the different gold methods, of which the following are the principal conclusions:

(1) The method of Löwit is particularly applicable to thin entire (unteased) muscles.

(2) Preliminary treatment with weak (0·5 per cent.) formic acid, and reduction in dilute glycerin containing one-fourth to one-fifth volume of formic acid, in the dark, is a good process for muscles of warm-blooded animals.

(3) The same process, with omission of the preliminary acidification, is to be recommended for cold-blooded animals.

(4) The method of GOLGI (*Mem. delle R. Accad. di Sci. di Torino*, ii, 32) is applicable to all classes of objects. It is as follows:—Acidification with 0·5 per cent. arsenic acid, impregnation in 0·5 per cent. solution of double chloride of gold and potassium, and reduction in 1 per cent. arsenic acid in sunlight.

(5) A modification of the last (apparently due to MAYS) consisting in impregnation in a mixture containing 0·5 per cent. arsenic acid, 0·25 per cent. chloride of gold and potassium, and 0·1 per cent. osmium, and reduction as before, is stated to be particularly applicable to Reptilia. (This process has been further modified by MARSHALL [*Quart. Journ. Mic. Sci.*, 1890, p. 73; *Journ. Roy. Mic. Soc.*, 1890, p. 404], who impregnates [after previous immersion for a few seconds in 1 per cent. acetic acid] in a mixture of 20 parts 1 per cent. acetic acid, 4 parts 1 per cent. gold chloride, and 1 part 1 per cent. osmic acid. He had found that the arsenic acid of Mays' formula disintegrated the muscle-fibre.)

Kühne also remarks that teasing ought to be done in the gold solution. Specimens should be removed from the gold every few minutes, and from the reducing medium every hour, so that the right duration both of impregnation and reduction may be hit off. Preliminary acidification is unfavorable to the preservation of the arborescence, and the treatment with acids after impregnation is best abbreviated as much as possible. Mounting in formic acid glycerin is not favorable for the preservation of detail. This is best studied in arsenic acid. Entire muscles are best mounted in balsam.

See also an older method of MAYS in *Zeit. f. Biol.*, 1884, p. 449; *Zeit. f. wiss. Mik.*, 1885, p. 242.

Tendon.

671. Corpuscles of Golgi (RANVIER, *Traité*, p. 929).—Take the tendon of the anterior and superior insertion of the gemini muscles of the rabbit. Free it as far as possible from adherent muscle-fibres. Treat it according to the formic acid and gold method (§ 225), and after reduction of the gold scrape the tendon with a fine scalpel, in order to remove the muscle-fibres that mask the "musculo-tendinous organs."

672. Corpuscles of Golgi (in the tendons of the motores bulbi oculi) (*v.* MARCHI's methods, *Archivio per le Scienze Mediche*, vol. v, No. 15).—The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (Golgi's method, *supra*, § 670), and by the methods of MANFREDI, given in § 228.

Mount all these preparations in glycerin (balsam clears too

greatly). The methods only succeed completely during fine sunny weather.

673. Corpuscles of Golgi (Cattaneo, *Arch. ital. de Biol.*, x, 1888, p. 337).—The method here recommended is the arsenic acid method of Golgi that has been described above, § 670, *sub voce* "Kühne" (4).

See also Ruffini (*Atti R. Acc. Lincei Roma*, 1892, p. 442; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 237), who recommends the method of Fischer.

674. Corpuscles of Golgi (Ciaccio, *Mem. R. Acc. Sci. Bologna* (4), t. x, 1890, p. 301; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 507). —For Amphibia the usual gold methods are not satisfactory, because the ground-substance of the tendon takes the stain at the same time as the nerve-endings. Pieces of tendon should be put into 0·1 per cent. hydrochloric acid or 0·2 per cent. acetic acid until quite transparent. They should then be put for five minutes into a mixture of 0·1 per cent. gold chloride and 0·1 per cent. potassium chloride. After that they are put back into the acetic acid, and remain there for a day in the dark, and for two or three hours more in the sunlight. When they have become somewhat violet they are put for a day into 0·1 per cent. osmic acid, and finally mounted in Price's glycerin acidulated with 0·5 per cent. of acetic or formic acid.

675. The Methylen-blue Method.—I find no mention of the application of this method to the study of the relations of nerve and tendon, for which it would seem *a priori* to be peculiarly suitable.

Smooth Muscle.

676. Smooth Muscle—Isolation of Fibres (Schwalbe, *Arch. f. mik. Anat.*, 1868, p. 394).—Maceration in weak chromic acid solution (0·02 per cent. proved a generally useful strength). This is a better reagent than osmic acid, 1 per cent. acetic acid (Moleschott), weak sulphuric acid, pyroligneous acid (Meissner), 20 per cent. nitric acid (Reichert), 32 to 35 per cent. potash solution (Moleschott), as it preserves better than any of these the finer structure of the cells.

Gage's methods.—See *Journ. Roy. Mic. Soc.*, 1887, p. 327; and §§ 529, 540, and 544, *ante*.

Möbius, liquid for maceration of the muscle of *Cardium* (see above, § 539).

Ballowitz, muscle of Cephalopoda, see *Arch. f. mik. Anat.*, xxxix, 1892, p. 291; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 344.

677. Bladder of Frog, Innervation of (Wolff, *Arch. f. mik. Anat.*, 1881, p. 362).—A frog is killed and a solution of gold chloride of 1 : 20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, dissect away the attachments of the bladder, ligature the intestine above the bladder, and cut away the abdomen of the frog so as to have in one piece bladder, rectum, and hind legs. (All this time the bladder must be kept moist with weak gold solution.) The bladder and the rest are now put into gold solution of 1 : 2000 for four hours; the bladder is then excised, slit open, and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a pencil if necessary. Put for twenty-four hours into gold solution of 1 : 6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in common daylight. The muscles should be pale blue-red; medullated nerves dark blue-red; sympathetic nerves and ganglia carmine-red. Ranvier (*Traité*, p. 854) recommends one or the other of his two gold processes. The bladder of frogs should be carefully distended by injection of the lemon juice or gold chloride and formic acid through the cloaca.

The *methylen-blue* method would appear to be very much indicated for this subject, but has not been tried so far as I am aware.

678. Musculus dilatator Pupillæ (Dogiel, *Arch. f. mik. Anat.*, 1886, p. 403).—An enucleated eye is divided into halves, and the anterior one with the iris brought for some days into a mixture of two parts one-third alcohol and one part 0·5 per cent. acetic acid. The iris can then be isolated, and split from the edge into an anterior and posterior plate, and these stained according to the usual methods.

679. Iris (Koganei, *Arch. f. mik. Anat.*, 1885, p. 1).—The pigmented epithelium can be removed by brushing with a

small brush after prolonged maceration in solution of Müller. The pigment may also be bleached by chlorine water, which, however, should only be allowed to act for a few hours, until the pigment has become of a light brown; complete decoloration may be obtained by prolonging the reaction for twenty-four hours, but then the tissues suffer. (See *Journ. Roy. Mic. Soc.*, 1886, p. 874.)

See also CANFIELD, in *Arch. f. mik. Anat.*, 1886, p. 121; and DOSTOIEWSKY, *ibid.*, p. 91 (sections stained with hæmatoxylin and eosin, or [LIST, *Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 514] with Renaut's hæmatoxylic glycerin).

680. Stomach of Triton (see STILLING and PFITZNER, in *Arch. mik. Anat.*, 1886, p. 396).

681. Test for Smooth Muscle (RETTERER, *Comptes Rend. Soc. Biol.*, iv, 1887, p. 645; *Journ. Roy. Mic. Soc.*, 1888, p. 843).—If a specimen of tissue be fixed in a mixture of ten volumes of 90 per cent. alcohol and one volume of formic acid, well washed, and stained for twenty-four to thirty-six hours with alum-carmine, the cytoplasm of smooth muscle will be found to be stained red, whilst connective-tissue cells remain unstained, and are swollen.

CHAPTER XXIX.

RETINA, INNER EAR, NERVES.

Retina.

682. Fixation and Hardening.—For section cutting, the retina *of small eyes* is best prepared by fixing the entire unopened bulb with osmium vapour. According to RANVIER (*Traité*, p. 954) you may fix the eye of a triton (without having previously opened the bulb) by exposing it for ten minutes to vapour of osmium. The sclerotic being very thin in this animal, such a duration of exposure is generally sufficient. Then divide it by an equatorial incision, and put the posterior pole for a few hours into one-third alcohol.

Somewhat larger eyes, such as those of the sheep and calf, may be fixed in solutions without being opened. But it is generally the better practice to make an equatorial incision, and free the posterior hemisphere before putting it into the liquid.

The older practice was to use strong solutions of pure osmic acid; but most of the best recent work has been done with chromic mixtures.

KUHNT (*Jen. Zeit. f. Naturw.*, Bd. xxiv, H. 1, 1889, p. 177; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 65) finds that for isolation preparations the best way is to fix for twenty to twenty-eight hours in $\frac{3}{4}$ per cent. osmic acid, then put the preparation for fourteen days into water in a well-closed vessel, changing the water once at the middle of the time, and at the end of it bringing the preparation for three to four weeks into a mixture of 80 parts water, 12 parts alcohol, and 8 parts glycerin.

For sections he prefers fixing for two days in solution of Flemming, hardening for three days in alcohol, and imbedding in celloidin.

BARRETT (*Quart. Journ. Mic. Sci.*, 1886, p. 607) recommends fixing the entire unopened bulb in a liquid containing 0·2 per

cent. of osmium and $\frac{1}{6}$ per cent. of chromic acid in water, for twenty-four to thirty-six hours, with subsequent hardening for fourteen days in alcohol containing 2 per cent. of carbolic acid. He finds that the preservation of specimens so treated is far superior to that of specimens hardened in pure alcohol.

Schiefferdecker (*Arch. f. mik. Anat.*, 1886, p. 305) used either Müller's solution, or chromic acid of $\frac{1}{6}$ per cent., or a mixture of 1 part of wood-vinegar (Acetum Pyrolignosum) with 3 parts of distilled water, which he states gives particularly instructive preparations without any serious alteration of the elements.

Cuccati (*Mem. R. Accad. Sci. Ist. di Bologna ; Zeit. f. wiss. Mik.*, v, 1, 1888, p. 86) fixes large eyes (the lens and vitreous body having been removed) for two days in a solution of—

1 per cent. osmic acid . . .	14 grms.,
1 per cent. chromic acid . . .	25 „
Acetic acid	1 drop,

which should be renewed after the lapse of twenty-four hours.

See also Dennissenko (*Arch. f. mik. Anat.*, 1881, p. 395).

Dr. Lindsay Johnson writes me that he gets the best results by fixing for three minutes with a mixture of equal parts of acetic acid and 2 per cent. osmic acid, injected into the vitreous or the cavity of the eye. After that the eye is washed in alcohol for a quarter of an hour, and put for two hours into the mixture, § 78 ; it is washed in running water, and suspended in a large volume of 2·5 per cent. bichromate of potash for two days, then passed gradually through successive alcohols, beginning with 20 per cent., and ending with absolute.

Chievitz (*Arch. f. Anat. u. Phys., Anat. Abth.*, 1889, supp., p. 139 ; *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 511) uses nitric acid of 2·5 per cent. strength. Entire heads of frogs may be fixed in it for a couple of hours, washed out for a night in water, and put up in alcohol. Bulbi of birds should be opened in the acid, and the retina carefully exposed by removing the cornea, iris, lens, and vitreous, or by separating the sclera and choroidea from it. The duration of these operations affords a sufficient fixation.

Angelucci (Moleschott's *Untersuchungen*, &c., xiv, 1890,

p. 231 ; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 85) recommends that eyes be put for from half an hour to three hours into 3 per cent. nitric acid, then for ten days into solution of Müller, and lastly into alcohol.

683. Staining.—Dr. Lindsay Johnson, writing to me, recommends, above all things, the Ehrlich-Biondi mixture, either alone or with addition of nigrosin (see § 259). Used in the latter way, the nuclear cells are stained pale brown, and nucleoli a darker brown; whilst the Müller fibre layers, molecular layers, and rods are stained a beautiful green, varying from yellowish to bluish green according to thickness of specimen and strength and length of action.

Ramón y Cajal employs the rapid chromate of silver method of Golgi, as given below (§ 715).

Kuhnt (see last section) employs Pal's modification of Weigert's hæmatoxylin process.

Dogiel employs the methylen-blue method, as given in §§ 117, 119.

Schaffer (*Sitzb. k. Acad. Wiss. Wien*, xcix, 1890, 3, p. 110; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 227) recommends mordanting sections in 1 per cent. chromic acid for some hours, washing for a *short* time only with water, staining for twenty hours in Kultschitzky's acetic acid hæmatoxylin (*post*, § 697), and differentiating for twelve hours in Weigert's ferricyanide solution.

Krause (l. c., § 685) obtains instructive preparations by treating fresh retina with perchloride of iron or of vanadium in 1 per cent. solution, and then with a 2 per cent. solution of tannic or pyrogallic acid. These reagents only stain the granule layers and the nuclei of the ganglion-cells. The elements of the other layers may then be stained with Säurefuchsin, or some other anilin.

Lennox (*Arch. f. Ophthalm.*, xxxii, 1 ; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 408; and *Journ. Roy. Mic. Soc.*, 1887, p. 339) has been applying Weigert's hæmatoxylin method to the retina, with some remarkable results.

Cuccati (l. c., last section) stained with concentrated aqueous solution of Säurefuchsin, and mounted in balsam.

See also Bernheimer, *Sb. k., Akad. Wiss. Wien*, 1884; or *Journ. Roy. Mic. Soc.*, 1886, p. 167 ; and Ramón y Cajal, *Rev. trim. de Hist. Norm. y Path.*, i, 1888, p. 1 ; *Anat. Anz.*, 1889, p. 111 ; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 373, and vi, 2, 1889, p. 204.

684. Sections.—Some workers recommend celloidin ; but I see no reason whatever for not employing paraffin. Sections

may be mounted in dammar or (Flemming) in glycerin. Ramón y Cajal (l. c. last section) mounts in dammar or colophonium varnish without a cover-glass.

685. Dissociation Methods.—For maceration preparations you may use weak solutions (0·2 to 0·5 per cent.) of osmic acid for fixation, and then macerate in 0·02 per cent. chromic acid (M. Schultze), or in iodised serum (M. Schultze), or in dilute alcohol (Landolt), or in Müller's solution, or (Ranvier, *Traité*, p. 957) in pure water, for two or three days. Thin (*Journ. of Anat.*, 1879, p. 139) obtained very good results by fixing for thirty-six to forty-eight hours in one-third alcohol, or in 25 per cent. alcohol, and then staining and teasing.

Schiefferdecker (l. c. § 682) macerates fresh retina for several days in the methyl mixture, § 548.

Krause (*Intern. Monatsch. f. Anat. u. Hist.*, 1884, p. 225; *Zeit. f. wiss. Mik.*, 1885, pp. 140, 396) recommends treatment for several days with 10 per cent. chloral hydrate solution. Barrett finds that this process preserves the rods and cones admirably.

Inner Ear.

686. Schwalbe (*Beitr. z. Phys.*, 1887; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 90; *Journ. Roy. Mic. Soc.*, 1887, p. 840).—Fix (cochlea of guinea-pig) for eight to ten hours in "Flemming," wash in water, decalcify (twenty-four hours is enough) in 1 per cent. hydrochloric acid, wash the acid out, dehydrate, and imbed in paraffin.

Prenant (*Intern. Monatsschr. f. Anat. u. Physiol.*, ix, 1, p. 6; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 379).—For sections, open the cochlea in solution of Flemming or of Hermann, and fix therein for four to five hours. Avoid decalcification as far as possible, as it is inimical to good preservation of elements; but if necessary, take 1 per cent. palladium chloride. Make paraffin sections and stain with safranin, or with methyl violet B, or with anilin green and orange, or with Renaut's eosin-hæmatoxylin.

Isolation preparations of the stria vascularis may be made by putting a cochlea for a day into 1 per cent. solution of osmic acid, then for four to five days into 0·1 per cent. solution; the stria may then be got away whole.

687. Other Methods.—Waldeyer, Stricker's *Handb.*, p. 958 (decalcification either in 0·001 per cent. palladium chloride containing 10 per cent. of HCl, or in chromic acid of 0·25 to 1 per cent.).

Urban Pritchard (*Journ. Roy. Mic. Soc.*, 1876, p. 211).—Decalcification in 1 per cent. nitric acid.

Lavdowsky (*Arch. f. mik. Anat.*, 1876, p. 497).—Fresh tissues (from the cochlea) are treated with 1 per cent. solution of silver nitrate, then washed for ten minutes in water containing a few drops of 0·5 or 1 per cent. osmic acid solution, and mounted in glycerin.

Max Flesch (*Arch. f. mik. Anat.*, 1878, p. 300).

Tafani (*Arch. Ital. de Biol.*, vi, p. 207).

Politzer, "Die anatomische u. histologische Zergliederung d. menschlichen Gehörorganes," Stuttgart (Enke), 1889 (see *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 364).—The reviewer (Haug) here calls attention to the admirable qualities of *phloroglucin* as a decalcification agent for this object (see § 562).

Eichler (*Abh. d. math.-phys. Cl. d. k. Sächsischen Ges. d. Wiss.*, Bd. xviii, 1892, p. 311; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 380).—Detailed account of manipulations for injection of blood-vessels of the labyrinth.

Dr. Lindsay Johnson greatly recommends the Ehrlich-Biondi stain (see §§ 259, 683).

Nerves.

688. Weigert's **Method for Medullated Nerves** (*Fortschr. d. Med.*, 1884, pp. 113, 190; 1885, p. 136; *Zeit. f. wiss. Mik.*, 1884, pp. 290, 564; 1885, pp. 399, 484).—The ordinary methods of staining with hæmatoxylin depend on the production of an aluminium lake of hæmatoxylin. Weigert's method depends on the formation of another lake, a chromium or copper lake. In consequence of the formation of these lakes hæmatoxylin acquires the property of staining the myelin of nerves in a quite specific way.

In Weigert's process the formation of these lakes takes place in the tissue itself. The details of the process have been considerably modified, both by other workers and by Weigert himself. I give here the 1885 method, a knowledge of which is necessary to the understanding of the subsequent modifications. It is as follows:—The tissues are to be hardened in bichromate of potash (the solutions of Müller or Erlicki will do as well, so far as I know). The hardening need only be carried to the point at which the tissues have

acquired a *brown*, not a *green*, coloration (but green tissues may be used, provided they have once passed through the brown stage). The preparation is then (but this is not necessary) imbedded by infiltration with celloidin, and the celloidin block fastened on cork and hardened in the usual way. The hardened block is put for one or two days into saturated solution of neutral acetate of copper diluted with one volume of water, the whole being kept at the temperature of an incubating stove. By this treatment the tissues become green, and the celloidin bluish green. The mordantage of the tissues is now terminated, and the preparation may be kept, till wanted for sectioning, in 80 per cent. alcohol.

Sections are made with a knife wetted with alcohol, and are brought into a stain composed of—

Hæmatoxylin	0·75 to 1 part.
Alcohol	10 parts.
Water	90 „
Saturated solution of lithium carbonate	1 part.

(A trace of any other alkali may be added in the place of lithium carbonate. The object of adding a little of some base is to "ripen" the hæmatoxylin solution.)

The sections remain in the stain for a length of time that varies according to the nature of the tissues:—Spinal cord, two hours; medullary layers of brain, two hours; cortical layers, twenty-four hours.

They are then rinsed with water, and brought into a decolourising solution composed of—

Borax	2·0 parts.
Ferricyanide of potassium . .	2·5 „
Water	200·0 „

They remain in the solution until they are decoloured to the right degree—that is, until complete differentiation of the nerves (half an hour to several hours)—and are then rinsed with water, dehydrated with alcohol, and mounted in balsam. They may be previously stained, if desired, with alum-carmine for the demonstration of nuclei.

For very difficult objects, such as pathological nerves, the decolouring solution should be diluted with water, and the immersion in it prolonged. GELPKE (*Zeit. f. wiss. Mik.*, 1885, p. 489) states that for transverse sections of atrophied nerves the solution should be diluted with fifty volumes of

water, and the immersion be prolonged to twelve hours at the least; for longitudinal sections it should be diluted with ten volumes of water.

The process is applicable to tissues that have been hardened in alcohol or in any other way, provided that they be put into a solution of a chromic salt until they become brown, before mordanting them in the copper solution.

As above stated, it is not necessary that the mordantage be done in bulk with tissues imbedded in celloidin. Max Flesch (*Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 50) finds that this practice is unfavorable to subsequent staining with other reagents than hæmatoxylin, and prefers (following Lichtheim) to make the sections first, bring them on closet-paper into the mordant, and after mordanting bring them on a spatula into 70 per cent. alcohol, and thence into the stain.

In the process given above, a copper lake is formed in the tissues. In the earlier form of the process the mordantage with the copper salt was omitted, and the stain depended on the formation in the tissues of a chromic lake. The results were not quite so good, and the process may be taken to be superseded by the copper process. A modified form of the former, due to Max Flesch, has some advantages, and is given in the next section.

If very many large sections have to be prepared, and if the staining solution be thrown away after using, the process may be found somewhat expensive. The following method for regenerating the staining solution is given by Fanny Berlinerblau (*Zeit. f. wiss. Mik.*, 1886, p. 50) :—About 2·5 to 5 per cent. of baryta water is added to the used solution; it is well shaken and allowed to stand for twenty-four hours; carbonic acid (obtained from the action of crude hydrochloric acid on marble) is led through it, it is allowed to stand for twenty-four hours more, and then filtered.

Paneth (*ibid.*, 1887, p. 213) makes the stain with extract of logwood instead of pure hæmatoxylin. One part of commercial extract of logwood is dissolved in 90 parts of water and 10 of alcohol. To the filtered solution is added 8 drops of concentrated solution of lithium carbonate for each 100 c.c. Sections require from eighteen to twenty-four hours in the stain at the normal temperature.

Breglia (*ibid.*, vii, 2, 1890, p. 236; see also *Journ. Roy. Mic. Soc.*, 1890, p. 817) stains with liquid extract of logwood or Pernambuco wood, prepared by extracting 7 to 10 grms. of the wood for 5 or 6 days with 100 c.c. of alcohol of 90 to 95 per cent.

The results obtained by Weigert's method are most splendid. The blue-black nerves stand out with admirable boldness on a golden ground. The method is applicable to the study of peripheral nerves as well as to nerve-centres, and is likely to be of great utility in Vertebrate embryology.

Nerve-tissue is not the only tissue stained by the process, which can be usefully applied to lymphatic glands and to skin (see Schiefferdecker, in *Anat. Anz.*, ii, 1887, p. 680).

Modifications of Weigert's Method.

689. Max Flesch (*Zeit. f. wiss. Mik.*, 1884, p. 564).—Sections, made either by the celloidin process or otherwise, are put for a few minutes or longer into 0·5 per cent. chromic acid solution; they are then rinsed in water, and brought into the stain, where they take on a sufficient coloration much more rapidly than the tissues will if stained in the mass. Decoloration is done in the usual way. No stove is required in this process, and either brown or green material may be used. Flesch finds (l. c., 1886, p. 51) that this method is to be preferred to Weigert's copper method in all cases in which it is important to produce differential staining in nerve-cells, and especially in the study of peripheral ganglia, and also for producing differential staining of the medulla of central and peripheral nerves, whilst the copper process is most certainly superior for the demonstration of fine nerve-fibres.

690. Benda (*Verhandl. Physiol. Ges. Berlin*, 1885–6, Nos. 12, 13, 14; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 410; *Arch. f. Anat. u. Phys.*, 1886, p. 562).—The tissues are to be fixed and hardened for at least three days in saturated solution of picric acid, washed out for several days in water, hardened in alcohol, imbedded in paraffin, and sections made. (The treatment with water after picric acid seems to me to be most inimical to the good preservation of the tissues, and I cannot understand why Benda should not have used alcohol.) The sections are mordanted by treating them for a few minutes or hours with a salt of iron, for which purpose concentrated solution of ammonio-sulphate of iron is recommended. After being well washed in several changes of water, they are stained for about ten minutes in 1 per cent. aqueous solution of hæmatoxylin, then decoloured by treatment for about five minutes in 0·05 per cent. aqueous solution of chromic acid, rinsed in water, dehydrated, cleared, and mounted in balsam.

This method is stated to be peculiarly adapted for the demonstration of very fine fibres and their relations to ganglion-cells.

691. Hamilton (*Journ. of Anat. and Physiol.*, xxi, 1887, p. 444; *Journ. Roy. Mic. Soc.*, 1888, p. 1051) mordants his preparations with a copper sulphate solution, and makes sections by the freezing method, after imbedding in collodion.

Beevor (*Brain*, 1885, p. 227; *Journ. Roy. Mic. Soc.*, 1886, p. 898) gives a very slight modification of Weigert's original process.

692. Pal (*Wien. med. Jahrb.*, 1886; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 92; *Med. Jahrb.*, 1887, p. 589; *Zeit. f. wiss. Mik.*, 1888, p. 88) describes the following process:—After staining

in the hæmatoxylin solution the sections are washed in water (if they are not stained of a deep blue, a trace of lithium carbonate must be added to the water). They are then brought for twenty to thirty seconds into 0·25 per cent. solution of permanganate of potash, rinsed in water, and brought into a decolouring solution composed of—

Acid. Oxalic. pur. . .	1·0
Potassium Sulphite (Kalium Sulfurosum [SO_3K_2]) . .	1·0
Aq. dest. . . .	200·0

In a few seconds the grey substance of the sections is decolourised, the white matter remaining blue. The sections should now be well washed out, and may be double-stained with Magdala red or eosin, or (better) with picro-carmine or acetic-acid-carmine.

For further details as to the somewhat elaborate minutiæ of the process see the papers quoted, or Behrens, Kossel, and Schiefferdecker's *Das Mikroskop*, i, p. 199.

Beevor (*Journ. Roy. Mic. Soc.*, 1892, p. 898) says of this process that it is more difficult to carry out than Weigert's, and that it does not show the different forms of fibres so well, but is useful on account of the double stain.

693. The process of Pal has been modified by Schäfer (I quote from Beevor, l. c., not knowing where the method was first published) as follows :—"The material is hardened from four to six weeks, and after cutting the sections are put into Marchi's fluid (1 part of a 1 per cent. solution of osmic acid, and 2 parts of a 3 per cent. solution of potassium bichromate). Then wash quickly in water and stain with the hæmatoxylin solution (hæmatoxylin 1 grm., acetic acid 2 c.c., water 100 c.c.). Develop afterwards as by Pal's method. This is a very good method for quickening the hardening process, and for sections which have been too long in alcohol after potassium bichromate."

694. Flechsig (*Arch. f. Anat. u. Phys., Physiol. Abth.*, 1889, p. 537; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 71; *Journ. Roy. Mic. Soc.*, 1890, p. 538) stains sections for three to eight days at a temperature of about 35° C. in a solution of redwood (Rothholz, Brazil-wood). This is prepared (after Branca) by dissolving 1 grm. of extract pur. of Japanese redwood in 10 grms. of absolute alcohol, and adding 900 grms. of water, 5 grms. of saturated solution of Glauber's salt, and 5 grms. of saturated solution of tartaric acid.

The sections are then washed in water and differentiated by the method of Pal.

Breglia (l. c. in last section) mordants sections for ten to fifteen minutes in a mixture of 15 c.c. of 90 per cent. alcohol with 3 to 7 c.c. of saturated aqueous solution of neutral acetate of copper (Armanni). They are then brought for five or ten minutes into a mixture of 3 parts of water with 1 part of saturated aqueous solution of lithium carbonate. They are then stained for eighteen to twenty-four hours in the extract of logwood solution given in the last section, to which if desired one-fifth to one-third of the lithium carbonate solution may be added. They are then differentiated in Weigert's decolourising solution. Instead of the acetate of copper, you may take a 4 to 7 per cent. solution of sulphate of copper, and mordant therein for twenty minutes; or carbonate of copper, subacetate of lead, perchloride of iron, or other salts may be used.

695. Vasale (*Rivista sper. di Freniatria*, xv, 1889, p. 902; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 517) finds that Weigert's process can be considerably abbreviated as follows. He stains *first* for three to five minutes in the hæmatoxylin, then treats the sections for the same length of time with saturated aqueous solution of neutral acetate of copper, in which they become black. They are then washed with water and differentiated by moving them about in Weigert's decolourising liquid diluted with one-half of water.

696. Another rapid modification is that of Lissauer, described by Sachs (*Centralb. f. Nervenheilk.*, xv, 1892, p. 330; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 391). Sections of material hardened in liquid of Müller are heated (the mordantage with copper being omitted) in 1 per cent. solution of chromic acid until the solution begins to form bubbles. They are then rinsed with water, and again heated, until bubbles begin to form, in Weigert's hæmatoxylin solution. They are then differentiated by Pal's process. The method is said to succeed even with material that has not been very well hardened, and to give good results with brains that have been only a short time in liquid of Müller.

697. Kultschitzky (*Anat. Anz.*, 1889, p. 223, and 1890, p. 519; *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 196, and vii, 3, 1890, p. 367) has given two forms of his well-known process, of which the following is the later:—Specimens are hardened for one to two months in solution of Erlicki, imbedded in celloidin or photoxylin, and cut. Sections are stained for from one to three hours, or as much as twenty-four, in a stain made by

adding 1 grm. of hæmatoxylin dissolved in a little alcohol to 100 c.c. of 2 per cent. acetic acid. They are washed out in saturated solution of carbonate of lithium or sodium. By adding to the carbonate of lithium solution 10 per cent. of a 1 per cent. solution of red prussiate of potash, and decolourising therein for two or three hours or more, a finer differentiation is obtained. After this the sections are well washed in water and mounted in balsam.

698. Rossi (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 182) also recommends a process stated to be simpler than Weigert's. The tissues are hardened in a liquid composed of water, 100 c.c.; chromic acid, 0·75 grm. to 1 grm.; acetate of copper, 5 grms. Human cord requires six to eight days; the cord of the dog, three to four; brain of dog, fifteen to eighteen. They are then dehydrated and imbedded in celloidin. Sections are stained in a liquid made by adding 7 to 8 drops of a 5 per cent. solution of hæmatoxylin in absolute alcohol to about 30 c.c. of alcohol. After two hours they are brought into a mixture of 8 drops of hydrochloric acid with 100 c.c. of absolute alcohol, and washed therein until the white substance is seen to be differentiated from the grey. They are washed out for twenty minutes or more in water, dehydrated, and mounted. The sections may be double-stained with borax-carmine.

699. Wolters (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 466) proceeds as Kultschitzky, except that he stains in a solution containing 2 grms. of hæmatoxylin (dissolved in a little alcohol) to 100 c.c. of 2 per cent. acetic acid, and kept warm by placing it on the top of a stove kept at 45° C. for twenty-four hours, after which time the sections are dipped in solution of Müller, and differentiated by the method of Pal. This is an intense *myelin* stain; medullated fibres blue-black; ground light; ganglion-cells yellow.

700. A second method of Wolters, which gave good results with the cerebellum and some peripheral nerves, is as follows:—Sections from material hardened in Müller and cut in celloidin are mordanted for twenty-four hours in solution of acetate of aluminium, or, which is better, in a mixture of—

10 per cent. vanadium chloratum . . 2 parts,
8 per cent. aluminium aceticum liquidum . 8 „

washed for five or ten minutes in water, stained in the above-mentioned hæmatoxylin, and differentiated in Weigert's decolourising mixture. A *myelin* stain in general, with superb differentiation of the protoplasmic processes of the cells of Purkinje.

For details of the numerous mordants that were unsuccessfully tried, see the paper quoted.

Wolters's *axis-cylinder* stain is given in § 726.

701. Mercier (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 480) has a modification which consists in adding glycerin to the stain (which possibly may serve to usefully increase its penetrating power), and employing after the usual differentiating fluid a second differentiating fluid composed of 2 parts of 10 per cent. potash solution, 10 parts of water, and 1 part of sulphuric ether. I cannot find that this process has been recommended by any other worker.

702. Haug's modification of Weigert's process is given in § 755.

703. Weigert's **New Method** (*Deutsche med. Wochenschr.*, 42, 1891, p. 1184; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 392).—The material is to be hardened in bichromate and imbedded in celloidin in the usual way. The hardened blocks of celloidin are brought into a mixture of equal parts of a cold saturated solution of neutral acetate of copper and 10 per cent. aqueous solution of potassio-tartrate of sodium ($C_4H_4O_6KNa + 4H_2O$, salt of Seignette). They are left in the mixture for twenty-four hours in an incubator. (Large specimens [Pons] will require forty-eight hours, the mixture being changed for fresh at the end of twenty-four hours.) They are then brought for twenty-four hours into aqueous solution of neutral acetate of copper, either saturated or diluted with 1 volume of water, being kept as before in the incubator. They are then rinsed with water and brought into 80 per cent. alcohol, in which they may either remain till wanted or be cut after half an hour.

The sections are stained for from four to twenty-four hours at the temperature of the room in a freshly prepared mixture of 9 vols. of (A) a mixture of 7 c.c. of saturated aqueous solution of carbonate of lithium with 93 c.c. of water, and 1 vol. of (B) a solution of 1 grm. of hæmatoxylin in 10 c.c. of alcohol (A and B may be kept in stock, but A must not be too old). The sections must be loose ones, not such as have been seriated in celloidin, and must not be thicker than 0·025 mm. The stain is poured off and the sections are washed in several changes of water poured on to them. They are then treated with 90 per cent. alcohol followed by carbolic-acid-and-xylol mixture (for a short time only), or by a mixture of 2 parts of anilin oil with 1 of xylol, then pure xylol and xylol balsam (not chloroform balsam, which injures the stain).

Medullated fibres dark blue on a light, sometimes rosy ground. If it be wished to have the ground particularly colourless, take instead of the second wash-water a mixture of $\frac{1}{5}$ to $\frac{1}{2}$ volume of common (not glacial) acetic acid with 100 volumes of water. Thick sections or series in celloidin require a special differentiation. They may be differentiated either with the above-mentioned acetic acid mixture, or in the usual borax-ferricyanide mixture diluted with water. In the latter case the ground will be yellow.

If the impregnation with the copper be imperfect (as, for instance, may happen if the treatment with the copper salt be performed at the normal temperature instead of in an incubator) some instructive differentiations of ganglion-cells may be obtained, the processes of the cells of Purkinje in the cerebellum, for instance, being very sharply brought out; but such preparations have a tendency to after-blackening, which does not happen with those that have been thoroughly impregnated with the copper.

The advantages of the improved method are that differentiation after staining is not necessary; that the annoying precipitates formed on the surface of the preparations by the copper in the old method do not appear; that the divers manipulations are simpler and easier; the preparations are equal in beauty to those of Pal, and can be obtained with greater certainty.

704. Golgi's **Nerve Methods—Bichromate and Silver Nitrate Process, or "Slow" Method.**—I take the following *résumé* of this method from the interesting paper of Golgi's pupil, Rezzonico, "Sulla struttura delle fibre nervose del midollo spinale," in the *Archivio per le scienze mediche*, iv, No. 4 (1879), p. 85.

1. Take pieces of perfectly fresh spinal cord, and soak them in a 2 per cent. solution of bichromate of potash for a period of time varying according to temperature. (In summer eight to fifteen days may suffice, in winter about a month is necessary.)

2. Wash them, and put them into a 0·75 per cent. solution of nitrate of silver. The period of immersion therein depends on the temperature: in summer the reaction will be complete throughout the tissues in two or three days; in winter, eight, ten, or more days.

3. Dehydrate small pieces with alcohol (make sections if necessary), clear in oil of turpentine, tease in the turpentine, and mount in dammar.

4. The preparations are then left to themselves in order that the secondary impregnation may take place. In direct sunlight eight to ten days will complete the process; in diffused daylight (or in the dark ?), twenty, thirty, or forty days.

A somewhat greater precision of the reaction is obtained by treating the fresh tissues with osmic acid (by means of insterstitial injection) before putting them into the bichromate. In this case a much shorter immersion in the bichromate will suffice (four, six, or eight days).

By this means may be demonstrated in the medullated fibres of the spinal cord a chain of conical funnels, set one within another, and embracing the axis-cylinder with their narrow aperture, and the external surface of the following funnel with their greater aperture; and it is seen that they consist of a fine spiral fibre wound into the form of a funnel. (The appearance of rings and strainers is due to imperfect action of the silver.)

705. For the Study of Peripheral Nerves Golgi modifies the process as follows (l. c., 1879, p. 238):

1. Pieces of nerve are immersed in the bichromate solution for from four, six, or eight hours to one day, or at most two days.

2. From time to time pieces are removed into the nitrate of silver; they remain there for from twelve to twenty-four hours.

3. They are washed with several changes of alcohol.

4. Tease in the alcohol, dehydrate, clear with turpentine, mount in dammar.

5. Reduce in direct sunlight; in summer a few days suffice, in cold weather some weeks are necessary.

Does not give quite such fine results as the osmium bichromate and silver method, or "rapid" method, next to be described; *but the preparations keep indefinitely.*

706. The Osmium, Bichromate, and Silver Method, or Rapid Method (*ibid.*, p. 237).—A perfectly fresh piece of nerve is thrown into the following liquid:

2 per cent. solution of bichromate of potash	10 parts.
1 per cent. solution of osmic acid .	2 „

After about an hour's immersion the piece of nerve may

be cut into lengths of ½ to 1 cm., which are put back into the liquid.

Four hours after the first immersion of the nerve in the mixture begin to put the pieces into nitrate of silver solution, transferring a certain number of pieces every three hours, so as to be sure that some of them shall have had a bichromate bath of a proper duration. (This duration may, roughly speaking, be said to lie between six and twenty-four hours.)

The strength of the nitrate of silver solution is 0·50 per cent. The duration of the silver-bath must not be less than eight hours; it may be indefinitely protracted.

Dehydrate, clear with turpentine, mount in dammar.

The method is *more expeditious and easier of application* than the bichromate and silver nitrate method, and the results are somewhat *more precise*, but *the preparations do not keep in dammar* (without special precautions, to be discussed below).

These two methods serve for the demonstration in *peripheral* medullated nerve-fibres of the funnel-shaped coils of sustaining filaments discovered by Rezzonico in the medullated fibres of the spinal cord.

In all methods for the demonstration of the funnels it is important to observe the utmost delicacy of manipulation, and in particular, the fibres *must not be stretched;* their stretching is a weak point in the methods of Ranvier (*Traité*, p. 718).

Modifications of Golgi's Chromate of Silver Method.

707. Critique of Golgi's Method.—The above-described methods have been found extremely valuable in the most various departments of nervous anatomy. They have given brilliant results in the study of peripheral nerves and their origins or terminations, and in the study of the relations of fibres and cells in the central nervous system. It has been found, at the same time, that they have the defect of considerable uncertainty in the production of the desired reaction, and a still greater uncertainty in the preservation of the stain. These defects have given rise to a most elaborate discussion, which unhappily has not as yet led to very satisfactory results.

Golgi's method is apparently (but this is by no means certain) a double decomposition method, based on the combination of the bichromate of potassium in the tissues with

the silver nitrate, and formation of a brown precipitate of bichromate of silver ($K_2Cr_2O_7 + 2AgNO_3 = Ag_2Cr_2O_7 + 2KNO_3$; the precipitate is brown by reflected light, but appears black by transmitted light). The problem is to preserve this precipitate in the tissues free from chemical or molecular change. And the problem is not an easy one; without special precautions the preparations will not resist the processes necessary for imbedding, will not always resist those necessary for merely mounting in balsam, and even then may easily "go bad" after they have been mounted for a short time.

708. SEHRWALD, in an elaborate paper (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 443), develops the theory that the deterioration of the preparations is due to solution of the precipitate in the reagents employed in the ulterior processes of preparation—in water, in chlorides (which may be present in alcohol), in the xylol, in the paraffin, in the very balsam itself. He made elaborate attempts (fruitless, but instructive) to turn the difficulty by transforming the soluble chromic salt of silver into some insoluble compound. He tried to replace the acid of the salt; he tried to replace the metal of the salt; he tried to make a sulphide of it; he tried to reduce it to the metallic state with photographic developing solutions (and had some success with hydroquinone, but does not recommend the process on account of its giving rise to deceptive precipitates that simulate organised elements). None of these experiments gave the desired result; and the upshot of the paper is that it is better not to attempt to modify the state of the precipitate, but rather so to modify the preparation liquids as to reduce their solvent action on the precipitate. This may be done by supersaturating them with bichromate of silver before using. The powdered salt should be added to each of them in excess, including the clearing media, the medium used for fixing the sections to the slide, the paraffin itself, the solvent used for removing it, and the balsam, and made to dissolve by the aid of heat. By this means thin sections may be prepared and mounted in balsam without injury to the stain. Sehrwald does not say how long they will keep.

One of the annoyances of Golgi's process is that it frequently gives rise to the formation at the periphery of the preparations of voluminous precipitates that are destructive

of the clearness of the images. SEHRWALD (l. c., p. 456) finds that this evil can be avoided by putting the tissues into gelatin solution before bringing them into the silver-bath. A 10 per cent. solution of gelatin in water may be made. The tissues are imbedded in this in a paper imbedding box with the aid of a little heat (the gelatin melting at a sufficiently low temperature), and are brought therein into the silver-bath. After the silvering the gelatin is removed by warm water saturated with chromate of silver.

709. SAMASSA (op. cit., vii, 1, 1890, p. 26) points out that bichromate of silver is not soluble either in absolute alcohol, toluol, xylol, paraffin, or Canada balsam, nor otherwise chemically affected by them; and that therefore Sehrwald's explanation is untenable. He thinks that the true explanation of the deterioration of the preparations is that the precipitate forming the impregnation is little by little floated away from the tissues by the mechanical force of the diffusion currents set up on the passage of the preparations through the different reagents, and particularly those long-continued ones caused by the slow drying of balsam under a cover-glass. He recommends, therefore, simply that the preparations be preserved without a cover.

710. GREPPIN (*Arch. f. Anat. u. Entw., Anat. Abth.*, 1889, Supp., p. 55; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 66) finds that by means of hydrobromic acid, suggested by NEUMANN, preparations made by the slow method may be rendered sufficiently resistent to bear mounting under a cover. After silvering, sections are made with a freezing microtome and treated for thirty to forty seconds with 10 per cent. solution of hydrobromic acid, and may then be well washed in several changes of water and mounted in the usual way. If they be cleared in clove oil and exposed therein to sunlight for ten or fifteen minutes, they will take on a violet tone, and details will be more strongly brought out. It is sometimes well to treat them, after the 10 per cent. acid, with 40 per cent. acid for twenty to thirty seconds. They may also be treated by Pal's modification of Weigert's hæmatoxylin process.

711. OBREGIA (*Virchow's Archiv*, cxxii, 1890, p. 387; *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 97; *Journ. Roy. Mic. Soc.*, 1891,

pp. 536, 830: the *Journal* here appears to have been tracking itself in its own snow; the two reports are *literatim* identical, the second one being copied from the *Amer. Mon. Micr. Journ.*, 1891, p. 210, which took it *verbatim* from the *Journal's* first one) gives the following. Sections of silvered material are made, either without imbedding or after imbedding either in paraffin or celloidin, care being taken in either case not to use alcohol of a lower grade than 94 or 95 per cent. They are brought from absolute alcohol into a mixture of eight to ten drops of 1 per cent. solution of gold chloride with 10 c.c. of absolute alcohol, which should be prepared half an hour beforehand and exposed to diffused light until the sections are placed in it, when it should be put into the dark. After fifteen to thirty minutes therein, according to their thickness, the sections are quickly washed in 50 per cent. alcohol, then in water, then treated for five or ten minutes with 10 per cent. solution of hyposulphite of soda. They are lastly washed well with water, and may then be mounted at once in balsam under a cover, or if desired may be previously stained with carmine or hæmatoxylin, or Pal's modification of Weigert's process, or the like.

Obregia thinks that the reason why Sehrwald did not succeed in substituting gold for the silver in his preparations (vide *supra*) is that he took the gold salt in aqueous solution.

This method is also applicable to material treated by Golgi's sublimate process, § 760.

712. Fick (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 168) does not admit Samassa's theory of the deterioration of sections through diffusion currents (*supra*). He points out that bichromate of silver is soluble in water, especially with the aid of heat; and after an elaborate series of experiments, concludes that the water of the reagents or damp confined by the cover-glass is the cause of the ruin of the preparations. Watery fluids should be avoided, and sections should be mounted without a cover, or on a cover raised free of contact with the slide by means of wax feet, or the like. Or sections mounted without a cover may be later on provided with one if the balsam be first rendered perfectly anhydrous by careful heating.

This last method is also recommended by Huber (*Anat. Anz.*, vii, 1892, p. 587; *Journ. Roy. Mic. Soc.*, 1892, p. 707).

713. There remain to be mentioned some variations in the details of the process, chiefly connected with the strengths of the solutions to be used and the duration of the impregnation-baths.

Mondino (*Arch. per le Sci. Med.*, viii, p. 45; *Zeit. f. wiss. Mik.*, 1885, p. 547) recommends that the preparations be left in the bichromate mixture longer than directed by Golgi—viz. from one to eight days; and also that they be left for over twenty-four hours in the silver-bath.

For some minutiæ concerning the application of the bichromate and silver method to cerebro-spinal nerves, see Petrone, in *Internat. Monatschr. f. Anat.*, v, 1, 1888, or *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 238.

714. Ramón y Cajal, who has done a great deal of important work by Golgi's method, has always used the rapid process. In *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 332, he gives it as follows :—Fresh *muscle of insects* put into a mixture of 20 parts 3 per cent. bichromate solution and 5 parts 1 per cent. osmium solution for twelve to twenty-four hours, then into 0·75 per cent. nitrate of silver for one day, then alcohol of 40 degrees (presumably Baumé, *i. e.* about 90 per cent.), then clove oil followed by resinified turpentine and (if I understand rightly) balsam. For the times and strengths used by him in his researches on the *cerebral cortex* of mammals, see his paper in *La Cellule*, vii, 1891, p. 125, or *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 239; also *Journ. Roy. Mic. Soc.*, 1892, p. 154. He generally hardened for from five to eight days at a temperature of about 25° C., and found it useful to adopt Sehrwald's gelatin process (*supra*, last section) for avoidance of peripheral precipitates. He prefers not to adopt Greppin's treatment with hydrobromic acid, nor Obregia's treatment with gold chloride, finding that although they serve to render the preparations permanent, they obscure the finer relations of fibres.

For embryos of the *fowl* he employs the same process; see his paper in *Anat. Anz.*, v, 1890, p. 85, or *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 235.

715. In a paper on the structure and relations of the *sympathetic ganglia* (which I have not seen, and quote from *Zeit. f. wiss. Mik.*, l. c.) Ramón y Cajal describes a process of "intensive" or "double" impregnation. After hardening for

three days (embryos of fowl) in the osmium-bichromate mixture, the preparations are put for thirty-six hours into nitrate of silver solution (0·5 to 0·75 per cent.). They are then brought back into the same osmium-bichromate mixture, or into a weaker one containing only 2 parts of osmic acid solution to 20 of the bichromate. After treatment with this they are washed quickly with distilled water, and put for a second time into the silver solution for thirty-six to forty-eight hours. It is important to hit off the proper duration of the first impregnation in the bichromate. If it has been too long (four days) or too short (one day), the second impregnation will not succeed. In this case a third impregnation must be resorted to, the objects being again treated with the weak osmium-bichromate mixture, and afterwards again with the silver solution.

For *retina* the process is given as follows :—Put the retina (batrachians and reptiles) with the sclerotic for twenty-four hours into the weak osmium-bichromate mixture. Then put it into 1 per cent. silver solution for twenty-four hours, then back into the osmium mixture for twenty-four hours, then for twenty-four hours again into the silver-bath.

716. Oyarzun (*Arch. f. mik. Anat.*, xxxv, 1890, p. 380) used the first-given process of Ramón y Cajal in his researches on the *fore-brain of Amphibia*. He gives the times as twenty-four hours in the osmium mixture, and the same in the silver-bath. This is for frogs. For Triton and Salamandra twenty hours is enough. Thirty to forty hours is too much.

717. Van Gehuchten (*La Cellule*, vii, 1891, p. 81 ; viii, 2, p. 225; see *Journ. Roy. Mic. Soc.*, 1892, p. 153, and *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 237), who has done some very important work on nerve-centres with Golgi's process, also follows the first-given modification of Ramón y Cajal, with this exception, that he employs formic acid to assist the action of the silver-bath (as suggested by Ramón y Cajal in an early paper). He treats the tissues (spinal cord and cerebellum in small pieces) with the osmium mixture for two to two and a half days at the ordinary temperature (or for less time at a temperature of 35° to 40° C.). They are then brought into the silver-bath, to which is added a little formic acid (1 to 2 c.c. to a litre, or 1 drop to 100 c.c. will do; care must be taken

not to add too much). Twenty-four hours in the bath is enough, but preparations may stay in it longer (in one case as much as two months) without hurt, provided they be kept in the dark. Wash, treat with alcohol for a quarter of an hour, then for the same time with absolute alcohol, and the same time with celloidin solution, after which they are hardened for about the same time in 70 per cent. alcohol, cut and mounted as usual.

718. Sala (*Zeit. f. wiss. Zool.*, lii, 1, 1891, p. 18; *Zeit. f. wiss. Mik.*, viii, 3, p. 389), in a paper written in Golgi's laboratory, gives the rapid process as follows (for the *pes hippocampi majoris* of small mammals) :—Four to five days in 2 per cent. bichromate of potash; twenty-four to thirty hours in a bichromate and osmium mixture containing 8 parts of the bichromate solution to 2 of the osmium solution; and a silver-bath of 0·75 per cent. strength. He finds Greppin's hydrobromic acid variation not merely useless, but hurtful. And he thinks that Sehrwald's process for imbedding the material in paraffin with the object of getting very thin sections is a mistake. The chief quality of Golgi's process is that it admits of the following of nerve-cell processes for a very great distance. Evidently this cannot be done with very *thin* sections. It is better simply to wash the preparations taken from the silver-bath with water, fix them to a cork with gum, put the whole into alcohol for a few hours to harden the gum, and cut with a microtome without imbedding.

Other Nerve Methods.

719. Structure of Medullated Nerve.—In order to demonstrate the axis-cylinder and the sheath of Schwann, the myelin may be removed. This may be done by boiling in caustic soda, and then neutralising; by boiling in a mixture of absolute alcohol and ether, and adding caustic soda; by boiling in glacial acetic acid; by boiling in fuming nitric acid, and adding caustic potash; or by treating with eau de Javelle.

720. Neuroceratin Structures (Galli, *Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 467).—An ischiatic nerve is excised, and fixed and hardened for eighteen to twenty minutes in solution of Müller. Small portions of the nerve are then further treated for one or two days with solution of Müller diluted with 2 parts of water, then for a quarter of an hour with glycerin containing 1 or

2 drops of glacial acetic acid for each cubic centimetre, and finally (without previous washing with water) are stained for fifteen to twenty minutes in aqueous solution of China blue (the best China blue for this purpose is that supplied by the Badische Anilin- und Soda-Fabrik, at Stuttgart). They are washed out in alcohol, cleared in essence of turpentine, and mounted in dammar.

For the results see the paper and the plate, l. c.

PLATNER (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 186) obtains a specific stain of the neuroceratin framework of medullated nerve in the following way :— Small nerves are fixed and hardened for several days in a mixture of 1 part of Liq. Ferri Perchlor. (Ph. G. Ed. ii) and 3 to 4 parts of water or alcohol. They are then to be washed out in water or alcohol till no traces of iron remain in them (the reaction of the washings with rhodanide of potassium being a good test of this), and are stained for several days or weeks in a concentrated solution of "Echtgrün" (dinitrosoresorcin, not "dinitroresorcin," as erroneously in Platner's paper) in 75 per cent. alcohol ; after which they are dehydrated, imbedded, and sectioned.

See also the papers of GEDOELST in *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126 (good details of digestion methods) ; also the report in *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 57.

Further, in general, RANVIER, *Traité*, p. 718, *et seq.* ; REZZONICO, *Arch. per le Sci. Med.*, 1879, p. 237 ; TIZZONI, *ibid.*, 1878, p. 4 (a process of boiling in chloroform for an hour or two, then staining and mounting in glycerin) ; BOVERI, *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 91 ; JAKIMOVITCH, *Journ. de l'Anat.*, xxiii, 1888, p. 142, or *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 526 (instructions for impregnating the axis-cylinder with silver, followed by reduction in formic acid and amyl alcohol) ; SCHIEFFERDECKER, in BEHRENS, KOSSEL, u. SCHIEFFERDECKER, *Das Mikroskop*, Bd. ii, p. 227.

720a. MARCHI'S **Method for Degenerate Nerves.**—I have not been able to find out where this was first published, and take the following from the report by Schiefferdecker of a paper by EIJKMAN, in *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 350. Nerves are first hardened for a week in solution of Müller, and then put for a few days into a mixture of 2 parts solution of Müller and 1 part 1 per cent. osmic acid solution. The treatment with the chrome salt deprives the medullary sheath of normal fibres of the faculty of impregnating with osmium, whilst the degeneration products in diseased sheaths retain that faculty. In consequence the sheaths in normal nerves acquire a yellow coloration, those of degenerated tracts a black one.

Axis-cylinder Stains.

721. Amongst the chief methods for the study of the relations of axis-cylinders or non-medullated nerve-fibres are *the gold methods* given in Chaps. XII in Part I, and XXVII and XXVIII in Part II. The method of Freud for nerve-centres, which will be given in the next chapter, is also a good one for the study of the topography of peripheral nerves. But perhaps even more useful are the *methods of* Golgi given above, and the very important *methylen-blue impregnation methods* that have been described in Part I, Chap. IX. The following are also serviceable methods.

722. Kupffer's Method (*Sitzb. math. phys. Kl. k. Bayr. Acad. Wiss.*, 1884, p. 446 ; *Zeit. f. wiss. Mik.*, 1885, p. 106).—A nerve is stretched on a cork and treated for twenty-four hours with 0·5 per cent. osmic acid. It is then washed in water for two hours and stained for twenty-four to twenty-eight hours in saturated aqueous solution of Säurefuchsin ; after which it is washed out for from six to twelve hours (not more in any case) in absolute alcohol, cleared in clove oil, imbedded in paraffin, and cut. Sections are said to show the axis-cylinder as a bundle of fibrils (stained red) floating in an albuminous liquid.

723. Nissl's Method (*Münchener med. Wochenschr.*, 1886, p. 528 ; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 398).—Bichromate objects to be treated as follows : —Alcohol of 95 per cent. ; aqueous solution of Congo red, of 5 : 400 strength, seventy-two hours ; alcohol of 95 per cent., five to ten minutes ; alcoholic solution of nitric acid of 3 per cent. strength, six hours ; alcohol, five minutes ; clove oil ; balsam.

724. Alt (*Münch. med. Wochenschr.*, 1892, No. 4 ; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 81) stains for a couple of hours in solution of Congo in absolute alcohol, and washes out with pure alcohol. The results are said to be specially adapted to the study of peripheral axis-cylinders. Schiefferdecker, reporting on the method, does not recommend it. Squire (using a 2 per cent. aqueous solution) says it is one of the best stains he has tried.

725. Similar to this is one of the methods of Rehm (*Münchener med. Wochenschr.*, 1892, No. 13 ; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 390). Sections are stained for a few minutes in concentrated aqueous solution of Congo, washed in alcohol, and treated for ten minutes, until they become blue, with alcohol acidulated with hydrochloric or nitric acid, then cleared with origanum oil and mounted. A sharp axis-cylinder stain with considerable richness of other detail.

But Rehm prefers the following simple process :—Sections of alcohol-hardened material are placed for one to two days in an aqueous solution of hæmatoxylin of 0·5 per cent. strength, then washed out in aqueous solution of carbonate of lithia (strength not given) until no more colour comes away

from them, dehydrated and mounted. Axis-cylinders, cells, and processes, grey-black. The sections may be after-stained for a few minutes with 0·1 per cent. aqueous solution of Bismarck brown.

726. The method of WOLTERS for axis-cylinder and cell staining (his myelin stains have been given above as modifications of Weigert's) is as follows (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 471) :

The material (either central or peripheral nervous tissue) is *hardened in liquid of* KULTSCHITZKY, § 43, followed by alcohol, *as there described.* It is imbedded either in celloidin or paraffin, and cut. The sections are mordanted for twenty-four hours in the chloride of vanadium and acetate of aluminium mixture used for Wolters' myelin stain, *ante*, § 700, washed for ten minutes in water, and stained for twenty-four hours in the hæmatoxylin mixture, § 699. They are washed out until they are of a light blue-red colour (it is not possible to specify exactly the time required) in 80 per cent. alcohol acidulated with 0·5 per cent. of hydrochloric acid. Remove the acid thoroughly by washing with pure alcohol, dehydrate, clear with origanum oil, and mount.

A sharp axis-cylinder stain, myelin being coloured only if the differentiation in the acid alcohol is insufficient. It is applicable to peripheral nerves, and also to the central nervous system. In this, besides axis-cylinders, nerve-cells and their processes are stained; pyramid cells, Purkinje's cells and their processes, and glia cells are sharply brought out. Fine images of epithelium-cells are also obtained; in a word, besides axis-cylinders, every species of cells present in the preparations are stained. This, and a superior certainty, are the advantages which Wolters claims for his method over that of GOLGI, to which it is in other respects comparable.

727. PALADINO'S **Iodide of Palladium Method** (*Rendic. R. Accad. Scienze Fis. e Mat.*, Napoli, iv, 1890, p. 14, and 1891 (1892), p. 227 ; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 237, and ix, 2, 1892, p. 238 ; *Journ. Roy. Mic. Soc.*, 1890, p. 817, and 1892, p. 439).—Pieces of material hardened in bichromate, chromic acid, or corrosive sublimate, and not more than 5 to 8 mm. in thickness, are put for two days into a large quantity (at least 150 to 200 c.c. for each piece) of 0·1 per cent. solution of

chloride of palladium (which may be made as directed in § 52). They are next put for twenty-four hours into a 1 per cent. solution of iodide of potassium (the later paper quoted says the iodide solution should be of 4—100 strength, and that a relatively small volume of it should be taken, otherwise the iodide of palladium, which is rapidly formed in the tissues, may be again extracted by the liquid: small pieces of tissue should not remain in it for more than one or two hours). Dehydrate; imbed, if necessary, in paraffin by the chloroform method; mount in balsam.

A brown stain, being both a myelin stain and an axis-cylinder and cell-process stain, and applicable both to central and to peripheral nervous structures. It is very well spoken of.

CHAPTER XXX.

CENTRAL NERVOUS SYSTEM.

728. Introductory.—For small objects, such as the spinal cord and encephalon of very small Mammalia and of inferior Vertebrata, the ordinary methods of microscopic anatomy are very often sufficient. The cord and encephalon of Batrachia, for instance, may be treated as follows :—Corrosive sublimate, half an hour or so; alcohol of from 50 to 70 per cent., about two hours; then borax-carmine, or Mayer's paracarmine; paraffin; and sections mounted in balsam. The same organs of somewhat larger animals, such as the cat or rabbit, can also be prepared without recourse to any very special methods. They may be fixed and hardened in liquid of Erlicki, or other bichromate solution, well washed out with water, stained and imbedded, or first imbedded in collodion and stained after sectioning with alum-carmine, ammonia-carmine, or Heidenhain's hæmatoxylin, or, if it be desired to study the topography of nerve-fibres, with Weigert's hæmatoxylin. Even the cord of man may be treated by these methods; or rather, could be so treated if it did not almost always come into the hands of the anatomist in a state of *post-mortem* softening that necessitates special precautions in the hardening process.

But the voluminous encephala of man and the larger Vertebrates cannot be thus simply treated. They require specially modified methods for hardening, for the manipulation of sections, and for staining.

These methods have lately been described with great completeness in the works of Bevan Lewis (*The Human Brain: Histological and Coarse Methods of Research*, London, Churchill) and Obersteiner (*Anleitung beim Studium des Baues d. nervösen Centralorgane im gesunden u. kranken Zustande*, Leipzig, Toeplitz). These very welcome additions to the literature of the subject relieve me from the obligation of treating the subject with all the minuteness that might be desired by specialists; the more so as they show that so to treat the subject requires a volume, not a chapter.

Hardening.

729. Hardening by the Freezing Method.—This is in many cases a very good method, and in particular may be of service for the histological study of the cortex.

If it be desired to freeze an organ that has been already hardened by reagents, the freezing may be done by means of a freezing mixture of ice and salt; but in this case the preparation should first be penetrated by a

mucilaginous or gelatinous freezing mass (§ 316, *et seq.*), in order to avoid the formation of ice crystals in the tissues. But in the case of fresh tissue, the ether freezing method is to be preferred. This method allows of rapidly producing any desired degrees of hardness, and maintaining them or allowing them to diminish as occasion may require, and is perhaps the only method by which satisfactory sections of unhardened nerve-tissue can be obtained.

The sections should be floated on to water, treated for a minute on the slide with 0·25 per cent. osmic acid solution, and stained or otherwise treated as desired.

For a detailed description of these manipulations see Bevan Lewis's *The Human Brain.*

730. Generalities on Hardening by Reagents.—If large pieces of nerve-tissue are to be hardened, it is necessary to take special precautions in order to prevent them from becoming deformed by their own weight during the process. Spinal cord or small specimens of any region of the encephalon may be cut into slices of a few millimetres thickness, laid out on cotton wool, and brought on the wool into a vessel in which they may have the hardening liquid poured over them. The wool performs two functions: it forms an elastic cushion on which the preparations may lie without being distorted by their own weight; and it allows the reagent to penetrate by the lower surfaces of the preparations as well as by their exposed surfaces. A further precaution, which is useful, is to hang up the preparations, lying on or in the cotton wool, in a glass cylinder or other tall vessel; by hanging them near the top of the liquid the processes of diffusion and the penetration of the reagent are greatly facilitated.

If the preparations are placed on the bottom of the vessel, they should never be placed one on another.

If it be desired to harden voluminous organs without dividing them into portions, they should at least be incised as deeply as possible in the less important regions. It is perhaps better in general not to remove the membranes at first (except the dura mater), as they serve to give support to the tissues. The pia mater and arachnoid may be removed partially or entirely later on, when the hardening has already made some progress.

The *spinal cord*, the *medulla oblongata*, and the *pons Varolii* may be hardened *in toto*. The dura mater should be removed at once, and the preparation hung up in a cylinder-glass,

with a weight attached to its lower end. The weight has the double function of preventing any part of the preparation from floating above the level of the hardening liquid (a thing that easily happens where somewhat dense liquids, such as Müller's solution, are used), and of preventing the torsions of the tissues that may otherwise be brought about by the elastic fibres of the pia mater and arachnoid.

The *cerebrum* should be very delicately laid out on a layer of cotton wool, or, if possible, hung up in it. Plugs of the wool should be put into the fissure of Sylvius, and between the operculum and the median lobe, and as far as possible between the convolutions. Unless there are special reasons to the contrary, the brain should be divided into two symmetrical halves by a sagittal cut passing through the median plane of the corpus callosum. Betz recommends that after a few hours in the hardening liquid the pia mater should be removed wherever it is accessible, and the choroid plexuses also. I have found this by no means easy, and think it is an operation that can only be recommended for experienced hands.

The cerebellum should be treated after the same manner.

The temperature at which the preparations are kept in the hardening solution is an important point. The hardening action of most solutions is greatly enhanced by heat. Thus Weigert (*Centralb. f. d. med. Wiss.*, 1882, p. 819; *Zeit. f. wiss. Mik.*, 1884, p. 388) finds that at a temperature of from 30° to 40° C. preparations may be sufficiently hardened in solution of Müller in eight or ten days, and in solution of Erlicki in four days; whilst at the normal temperature two or three times as long would be required.

But it is not certain that this rapid hardening always gives the best definite results. Sahli, who has made a detailed study of the hardening action of chrome salts, is of opinion that it does not, and thinks it ought for this reason to be abandoned (see *Zeit. f. wiss. Mik.*, 1885, p. 3).

On the other hand, the slowness of the action of chromic salts at the normal temperature is such that decomposition may easily be set up in the tissues before the hardening and preserving fluid has had time to do its work. For this reason voluminous preparations that are to be hardened in the slow

way should be put away in a very cool place—best of all in an ice-safe.

731. The Reagents to be employed.—The hardening agents most used are the chromic salts. Chromic acid was much used at one time, but most workers now agree that its action, though much more rapid than that of the salts, is much more uneven, and frequently causes a disastrous friability of the tissues. Osmic acid is excellent—according to Bevan Lewis, it is the best of hardening agents; but its employment is unfortunately very restricted, as it can hardly be used for objects of more than a cubic centimetre in size.

It has already been noted that the liquid of Erlicki has a more rapid action than the other solutions of chromic salts; for this reason it is one of the most commonly employed solutions. Sahli, however (l. c.), after having studied the action of the usual solutions, concludes that the best hardening agent for fresh tissues is pure bichromate of potash, in 3 or 4 per cent. solution, the hardening being done in a cold place. And he does not approve of the addition of sodium sulphate (Müller), and rejects the liquid of Erlicki on account of the precipitates it so frequently gives rise to (see § 77).

Obersteiner is of the same opinion, recommending pure bichromate for general hardening purposes; whilst for the study of the most delicate structural relations he recommends fixing in Fol's modification of Flemming's liquid (§ 35) for twenty-four hours, followed by washing with water and hardening in 80 per cent. alcohol.

In view of the slowness of penetration of chromic salts it is often advisable to treat preparations for twenty-four hours or more with alcohol of 80 to 90 per cent. before putting them into the hardening liquid, in order to avoid maceration of the deeper layers of tissue.

For the question as to how far certain so-called pathological alterations of ganglion-cells should be attributed to putrefactive changes or to the influence of reagents see *Neurologisches Centralb.* for the years 1884 and 1885.

As to the so-called "pigment spots" produced by the liquid of Erlicki see *supra*, § 77.

732. Strengths of the Reagents.—All hardening reagents (except osmic acid) should at first be taken as weak as is con-

sistent with the preservation of the tissue, and be changed by degrees for stronger.

Osmic acid may be taken of 1 per cent. strength, and will harden small pieces of tissue sufficiently in five to ten days (EXNER).

Bichromate of potash should be taken at first of not more than 2 per cent. strength; this is then gradually raised to 3 or 4 per cent. for the cord and cerebrum, and as much as 5 per cent. for the cerebellum. Obersteiner begins with 1 per cent., and proceeds gradually during six to eight weeks to 2 or 3 per cent. (This is at the normal temperature; at a temperature of 35° to 45° C. the hardening can be got through in one or two weeks.)

Bichromate of ammonia should be taken of half the strength recommended for bichromate of potash, or even weaker at first; it may be raised to as much as 5 per cent. for cerebellum towards the end of the hardening.

Chromic acid is not much used alone. See § 69. It forms part of some of the mixtures mentioned below. A very little chromic acid (say one to two drops of 1 per cent. solution for each ounce) added to bichromate solution will do no harm, and will quicken the hardening.

Nitric acid has been and still is employed in strengths of 10 to 12 per cent., and gives particularly tough preparations. Perhaps a weaker solution might give good results, but I cannot find that any such have been tried.

Neutral acetate of lead in 10 per cent. solution affords an excellent preservation of ganglion-cells, according to ANNA KOTLAREWSKI (see *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 387).

TRZEBINSKI (*Virchow's Arch.*, 1887, p. 1; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 497) finds that as regards the faithful preservation of ganglion-cells (of the spinal cord of the rabbit and dog) the best results are obtained by hardening for eight days in 10 per cent. solution of corrosive sublimate, followed by hardening in alcohol containing 0·5 per cent. of iodine.

DIOMIDOFF (*ibid.*, p. 499) also obtained very excellent results by hardening small pieces of brain (as suggested by GAULE, OGATA, and BECHTEREFF) for from five to nine days (not more in any case) in 7 per cent. sublimate solution, and then putting the tissues for twenty-four hours into 50 per cent. alcohol, and for the same time into 70 per cent. and 96 per cent. alcohol successively. (This process produces artificial "pigment spots," similar to those produced by solution of Erlicki; they may be dissolved out by prolonged treatment with warm water, or in five minutes by strong solution of LUGOL.) The tissues are of a good consistence for cutting. Chloride of zinc has been recommended for some purposes (see below, § 780).

The next following paragraphs give in detail some methods of hardening recommended by some of the most competent workers.

733. Spinal Cord (KRAUSE, *Arch. f. mik. Anat.*, 1875, p. 226). —Solution of Müller, twenty-four hours. Then chromic acid, 1 per cent. On the fourth day the solution must be changed for fresh. A few days later (when the cord appears hard) the chromic acid is removed by means of water, and the cord put into spirit, followed by absolute alcohol, which must be changed at least twice.

CIAGLIŃSKI (*Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 19) recommends hardening for three or four months in solution of Müller, renewed every day during the first week, and afterwards as often as it becomes turbid. Liquid of Erlicki will harden in as many weeks, but has the defect that if preparations are left longer in it they are injured by the formation of the above-mentioned precipitates (last section and § 77).

734. Encephalon (M. DUVAL'S methods, ROBIN'S *Journal de l'Anatomie*, 1876, p. 497).—*First Method.*—Place the fresh tissues in solution of bichromate of potash 25, water 1000; change the liquid after the first twenty-four hours, and again after three or four days. After two or three weeks place the preparations in chromic acid of 3 per 1000, change the liquid every day for the first week, and after that every eight days until the middle of the second month, after which time it is no longer needful to change the liquid. The preparations must remain at least two months in the chromic acid; the longer they remain in it the better. A few fragments of camphor should be added to the liquid in order to prevent the growth of mould.

Second Method.—Place the fresh tissues in a mixture of equal parts of glycerin and acetic acid; after twenty-four hours remove them to Müller's solution, and after forty-eight hours more to chromic acid. (The strength of the solution is not indicated.) Change the chromic acid once or twice, and the preparations will be fit for cutting in about eight or ten days. (Small encephala [rat, bat] need not be extracted from the cranium provided this be largely opened before immersing them in the glycerin, nor need they be extracted before cutting, as the cranium will be found to be completely decalcified.)

This method is highly expeditious, and furnishes hardened tissues of a singularly homogeneous consistence, quite without fragility, but it should only be employed for the purpose of obtaining general views of structural relations, as the anatomical elements are somewhat changed by it: cells and axis-cylinders swell.

735. Cerebrum (Rutherford; from Bevan Lewis).—Place small portions of cerebrum in methylated spirit for twenty-four hours in a cool place. After that put them into the following solution:

Chromic acid	1 part.
Bichromate of potash . . .	2 parts.
Water	1200 ,,

Change the liquid at the end of eighteen hours, and then once a week. Should the tissue not be sufficiently tough for cutting at the end of six weeks place it in a $\frac{1}{6}$ per cent. solution of chromic acid for a fortnight, and then in rectified spirit.

736. Cerebrum (Bevan Lewis, *The Human Brain*, p. 102).—Methylated spirit, twenty-four hours in a cool place. Müller's solution, three days in a cool place. Then change the liquid; and after three days more change it again, or, preferably, substitute a 2 per cent. solution of potassium bichromate. At the end of the second week a solution of double the strength may be added; and if, at the termination of the third week, the mass is still pliable and of the consistence of ordinary rubber, it is as yet unfit for section cutting, and the reagent should be replaced by a solution of chromic acid.

737. Brain (Hamilton, *Journ. of Anat. and Physiol.*, 1878, p. 254).—Take a fresh brain and make a series of incisions into different parts, still keeping everything *in situ*; or slice it into any number of segments about one inch thick, but of the whole length or breadth of the organ, as may be desired. Do *not* remove the membranes; they form a protection for the superficial layers, and do not interfere with the hardening process. The large segments are placed flat in a large vessel padded with cotton; do not put them one above the other. Cover them with the following fluid:

Müller's fluid . . .	3 parts.
Methylated spirit . .	1 part.

(Heat is evolved on mixing these liquids, and the mixture must be allowed to cool before pouring it over the brain tissue.) Put the preparations away in an ice-safe. Turn the segments over next day. Change the solution in a fortnight or three weeks; or if on examining a section of one of the pieces it is found that the hardening reagent has penetrated

to the interior, they may be at once removed to the following mixture:

Bichromate of ammonia . .	1 grm.,
Water	400 c.c.,

in which they remain for one week. Then change the solution to one of 1 per cent. for one week; and let this be followed by a solution of 2 per cent. for another week, or longer if required. The pieces will now be sufficiently hard for cutting; they may be kept permanently in solution of chloral hydrate, twelve grains to the ounce.

Probably the chloral hydrate serves to attenuate the yellow coloration produced by the chromic liquids.

This is a process particularly adapted to the preparation of *large* segments of brain. The consistence is very tough and firm.

738. Entire Encephalon (Deecke, *Journ. Roy. Mic. Soc.*, 1883, p. 449).—"To harden the entire brain so that the inside and the outside shall be hardened equally and properly, Dr. Deecke finally adopted bichromate of ammonia in $\frac{1}{2}$ to 1 per cent. solution, according to the consistence of the brain. When nominally soft he adds say $\frac{1}{6}$ to $\frac{1}{10}$ per cent. of chromic acid to the solution, and always $\frac{1}{6}$ to $\frac{1}{4}$ of the whole volume of alcohol. It is then placed in a refrigerator and the fluid changed frequently. After a month add a little more alcohol from week to week until the alcohol is 90 per cent. This is changed as often as it is discoloured. The treatment requires from twelve to eighteen months."

739. Betz's **Methods** (*Arch. f. mik. Anat.*, 1873, p. 101).—The *spinal cord, medulla oblongata,* and *pons Varolii* are treated as follows:—The dura mater is removed, and they are hung up in a cylinder containing 75 to 80 per cent. alcohol, to which is added enough iodine to produce a light brown coloration. After from one to three days the preparation will be found to be somewhat surface-hardened; it is taken down, and the pia mater and arachnoid are removed. If the pia mater does not come away completely enough the preparation is put back for some days into the alcoholic iodine. The membranes having been removed, the preparation is put back into the original fluid, which is found to have become colourless owing to absorption of the iodine by the tissues. Fresh quantities of a strong solution of iodine in alcohol are from time to time added to the liquid in order to keep it at its original strength of iodine (as shown by the colour). If the membranes have been carefully removed it will be found that after about six days the preparation ceases to take up further quantities of iodine. The preliminary hardening may now be considered complete.

The preparation is now brought into a 3 per cent. solution of bichromate of potash. (A small weight is attached to it to prevent any portion of it from floating above the surface of the liquid. After a day or two it will have lost much of its alcohol, and will sink to the bottom of the vessel, which is equally undesirable; this must be watched for, and the preparation hung up or otherwise supported.) The vessel is put away *in a cool place.* As soon as a brown turbidity is seen in the liquid, together with a brown deposit on the preparation, the hardening may be considered to be complete. The preparation must be at once washed with water, and put away until wanted in a ½ to 1 per cent. solution of bichromate.

Cerebellum.—Must be quite fresh, and before placing in the iodine the membranes and vessels must as far as possible be very carefully removed. (If the pia mater does not come away freely, the organ must be macerated for a few hours in iodine solution in which other preparations have been kept, and which is diluted before using for this purpose.) The membranes having been removed, the cerebellum is placed (supported on cotton wool, with which the different organs are so propped up as to preserve their natural position) in solution of iodine for two or three days, and fresh iodine solution frequently added.

The pia mater is now removed from the rest of the preparation, which is put back for seven to fourteen days into the iodine solution. If at the expiration of this time it be found that the cerebellum can be supported on the finger by the vermiculus alone without bending, the preliminary hardening is complete, and it is brought into a 5 per cent solution of bichromate, where it remains until fit for cutting.

Cerebrum.—The cerebrum is divided into two halves along the median line of the corpus callosum, and put into the iodine solution. After a few hours the pia mater is removed from the fissure of Sylvius and from the corpus callosum, and if possible the choroid plexus is removed likewise.

The preparation is now put away in the iodine solution in a cool place (in summer in a cool cellar), and fresh iodine added as soon as the liquid is seen to lose colour (which must be watched for). After twenty-four to forty-eight hours the remaining pia mater is carefully removed by means of scissors and forceps from the fissures and convolutions, and one half-volume of fresh iodine solution is added to the liquid. (To facilitate the penetration of the liquid, wads of cotton wool are stuffed into the fissure of Sylvius, between the operculum and the median [central] lobe, in the direction of the descending cornu, and between the convolutions). After twenty-four to seventy-two hours the brain is brought into fresh solution of iodine in 70 per cent. alcohol, where it remains until the hemispheres are hard enough to be supported on two fingers without bending. (This will not be before ten to fourteen days.) It is then put into 4 per cent. solution of bichromate and left to acquire its definitive hardness. If an excessive brown deposit make its appearance, and the brain be found notwithstanding to be not hard enough for cutting, it must be rinsed with water and the bichromate solution changed. When ripe for cutting the brain ought to show an almost equal intensity of yellow-brown stain over the whole surface of a cut made through the total thickness of a hemisphere.

Brains that are not fresh require for hardening longer time and stronger alcohol.

Instead of the iodine solution, it is possible to use for the preliminary hardening a mixture of equal volumes of chloroform and ether; but this mixture is not to be recommended, on account of its solvent action on protoplasm and on the processes of ganglion-cells.

The methods of Betz are particularly adapted to the hardening of voluminous specimens, and of tissues that are in a state of post-mortem softening.

740. Osmic Acid (Exner, *Sitzb. k. Akad. Wiss. Wien*, 1881, lxxxiii, 3 Abth.; Bevan Lewis, *The Human Brain*, p. 105).—A small portion of brain, not exceeding a cubic centimetre in size, is placed in ten times its volume of 1 per cent. osmic acid. The solution should be replaced by fresh after two days, a proceeding which may advantageously be repeated at the end of the fourth day. In from five to ten days the piece is usually hardened throughout, and may be washed with water, treated with alcohol, and imbedded. The sections may be treated by a drop of caustic ammonia, which clears up the general mass of the brain substance, leaving medullated fibres black. B. Lewis says that this method exhibits a wealth of structure which no other method displays. The sections may be mounted in soluble glass. The chief value of this method is for tracing the course of medullated fibres.

741. Bellonci (*Arch. Ital. de Biol.*, vi, p. 405) employed this method in his researches on the optic nerve of mammalia. He used an osmic acid solution of 0·5 to 1 per cent., hardened for only fourteen to twenty hours, made sections, and treated them for three or four hours with 80 per cent. alcohol, and then with ammonia.

742. Nervous Centres of Reptiles and Batrachia (Mason, *Central Nervous System of Certain Reptiles*, &c.; Whitman's *Methods*, p. 196).—Iodised alcohol, six to twelve hours; 3 per cent. bichromate, with a piece of camphor in the bottle, and to be changed once a fortnight until the hardening is sufficient (six to ten weeks).

Burckhardt (*Das Centralnervensystem von Protopterus*, Berlin, 1892; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 347) recommends a liquid composed of 300 parts of 1 per cent. chromic acid, 10 parts of 2 per cent. osmic acid, and 10 parts of concentrated nitric acid, in which brains of Protopterus are hardened in twenty-four to forty-eight hours.

Imbedding and Cutting.

743. The Methods of Imbedding.—The paraffin infiltration method can only be used for the smaller objects of this class. Human spinal cord (which is quite at the upper limit as regards size) can be properly penetrated with paraffin by taking the precaution of first cutting it up into slices of not more than a few millimetres—preferably not more than one—in thickness. The largest objects of this class, such as entire

hemispheres of man, cannot be properly penetrated by any known imbedding mass; and the anatomist must be content with simple superficial imbedding—a proceeding which is here of the greatest service. For intermediate objects—those whose size varies between that of a small nut and a walnut—it appears to me that no hesitation as to the proper course is possible; such objects should be treated by the collodion method, which is at once the safest, the most convenient, and the most advantageous, as regards the ulterior treatment of sections.

Bevan Lewis recommends the ether freezing method for fresh brain. For hardened brain he recommends some of the old-fashioned wax-and-oil and other fatty mixtures, a doctrine at which I am surprised.

Hamilton recommends freezing in the gum and syrup mass given above, § 316. He also recommends a method of penetrating with collodion, which is hardened in the usual way, the hardened mass being cut with an ice-and-salt freezing microtome (see *Journ. of Anat. and Physiol.*, 1887, p. 444; or *Journ. Roy. Mic. Soc.*, 1888, p. 1051).

For sections of entire human brain, Deecke (l. c., § 738) proceeds as follows:—The brain to be cut is placed upon the piston of the microtome (a Ranvier model) and held *in situ* by several pieces of soft cork. It is then imbedded in a cast of paraffin, olive oil, and tallow, which, after it has become hard, is held in position by a number of small curved rods attached to, and projecting upwards from, the piston to the height of about an inch. Before cutting, and as it proceeds, the cast is carefully removed from around the specimen to the depth of about half an inch (which is easily done by the use of a good-sized carpenter's chisel), so that the knife never comes in contact with the cast.

Cutting is done under alcohol, the entire microtome being immersed in a copper basin. The sections are floated, with the aid of a fine camel-hair brush, on to sheets of glazed writing-paper. They are removed thereon successively into staining, washing, and clearing fluids. After clearing, they are brought on the paper on to a slide, and the paper is gently pulled away from them; they are then mounted in chloroform- or benzol-balsam.

It should be noted that the membranes should *not* be removed from the brain; they present no obstacle to cutting if this is done with a slight sawing movement, or with a series of short cuts, instead of one sweep of the knife. By this plan the sections are much more perfect and uniform in thickness, and the loss in a series of from four to five hundred to the inch through the entire cerebrum of man may not amount to more than 2 or 3 per cent.

Osborn (*Proc. Acad. Nat. Sci. Philadelphia*, 1883, p. 178, and 1884, p. 262; Whitman's *Methods*, p. 195) found advantage in employing Ruge's egg-mass (for the brain of Urodela). He recommends that the mass be injected into the ventricles.

The paraffin and collodion methods have already been sufficiently described in Part I.

Ciagliński (*Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 22) gives the following process for imbedding spinal cord :—Dehydrated segments of hardened cord are left for twenty-four hours in anilin oil, and then put for two or three hours into xylol. They are then brought into a mixture of equal parts of xylol and melted paraffin, and kept therein for twenty hours at a temperature of 30° C. They are then put through a twenty-four hours' bath of pure paraffin to which a few drops of cedar oil have been added (said to make the paraffin more elastic), the paraffin taken being of 45° C. melting-point for winter work, and 52° C. for hot summer weather. Lastly, they have a short bath of pure paraffin without cedar oil.

Feist (*ibid.*, viii, 4, 1892, p. 492) gives a useful hint for marking the right and left sides of spinal cord. He imbeds with each segment of the cord a small cylinder (of about 1 square millimetre in section) of hardened liver, stuck vertically in the imbedding mass (either celloidin or paraffin) against the side of the cord that it is desired to mark.

Staining.

744. The Kinds of Stains.—By a "general stain" is meant one by which it is intended to demonstrate as far as possible all the histological elements of a preparation.

It does not seem possible to divide the stains used in the study of the central nervous system accurately into the two categories of general and special. But an incomplete division is better than none at all, so I have grouped the methods as general and special, meaning by the latter term those that are principally intended for the demonstration of the relations of nerve-fibres and ganglion-cell processes, and that make no claim at all to the demonstration of other elements.

The nuclear stains that are so important in other branches of histology are here much less useful, partly because the nuclei of nerve-cells happen to be remarkably poor in chromatin, and partly because the chief point of interest is not the relations of nuclei, but the relations of fibres to one another and to cells.

A. *General Stains.*

745. *Ammonia-carmine* is old-fashioned, but may be used for general views. Beale's formula is a good one, especially where prolonged staining is required. But in view of the greatest precision of stain, the process of Gierke, or that of Betz, should be preferred (see § 165).

Picro-carmine is also an excellent reagent. It has much the same action

as ammonia-carmine, but gives a better demonstration of non-nervous elements.

Chromic objects stain very slowly in both these media. Sections may, however, be stained with them in a few minutes if they be put into a watch-glass with the stain, and the whole be kept on a wire-net over a water-bath heated to boiling-point (OBERSTEINER). See also § 163.

746. HENLE (*Handb. d. Nervenlehre*, 1871) gives the following, after MERKEL. In order to do away with the slowness of staining of tissues hardened in chromates, sections should be placed in solution of chloride of palladium (1 in 300 to 1 in 600) till they are of a straw colour (one or two minutes), rinsed in water, and stained in strong ammonia-carmine. Myelin, yellow; axis-cylinders, nerve-cells, and neuroglia, deep red.

747. SCHMAUS (*Münch. med. Wochenschr.*, 1891, No. 8; see *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 230, and *Journ. Roy. Mic. Soc.*, 1892, p. 439) obtains a ground-stain and axis-cylinder stain by means of a uranium soda-carmine solution.

748. UPSON (*Neurolog. Centralb.*, 1888, p. 319; *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 525) employs a stain made by adding to 5 c.c. of strong Grenacher's alum-carmine 1 to 3 drops of phospho-molybdenic acid. In this, sections stain in from two to ten minutes.

He also employs a stain made by saturating alum-carmine with zinc sulphate. Sections stain in this in from half an hour to twelve hours. This is said to give very good differentiations of the elements of peripheral nerves.

A third method of the same author consists in staining with carminic acid, and afterwards treating with a mordant. See the papers quoted.

FREEBORN (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 305) also recommends carminic acid, followed by treatment with 10 per cent. solution of chloride of iron.

749. *Borax-carmine* may be used. It is chiefly useful when employed for double-staining with indigo-carmine or an anilin blue to follow. I have obtained some superb stains with Seiler's borax-carmine and indigo-carmine process (§ 241). MERKEL's mixture of borax-carmine and indigo-carmine (§ 242) has been strongly recommended by MAX FLESCH (*Zeit. f. wiss. Mik.*, 1884, p. 566, and 1885, p. 349), who says that it gives extremely rich and instructive images.

DUVAL (*Journ. de l'Anat.*, 1876) speaks very highly of the double-stain quoted *ante*, § 243.

750. *Alum-carmine* (Grenacher's or Csokor's) may be used as a nuclear stain (OBERSTEINER). The stain principally takes effect on non-nervous nuclei.

751. REHM (*Münch. med. Wochenschr.*, 1892, No. 13; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 389) gives the following:—Sections (of alcohol-hardened material) stained for five minutes in 1 per cent. ammonia-carmine, and washed out in 70 per cent. alcohol acidified with 1 per cent of nitric acid; the acid removed by pure alcohol; the sections stained for half a minute

in 0·1 per cent. solution of methylen blue, differentiated in alcohol, cleared in origanum oil, and mounted in colophonium. Nuclei and axis-cylinders, red. This stain is said to prove that the nuclei of the ganglion-cells of the rabbit contain only one true nucleolus, instead of two or more, as has been hitherto believed.

752. For a plasma-stain REHM (l. c.) gives a method modified from NISSL. Sections of alcohol-hardened material are stained for half a minute to a minute in a *hot* 0·1 per cent. solution of methylen blue, washed in 96 per cent. alcohol till no more colour comes away, cleared with origanum oil, and mounted in balsam or benzin-colophonium. Nerve-cells, dark blue; connective-tissue cells lighter, and greenish.

753. To obtain a sharper distinction between nerve-cells and connective-tissue cells, REHM stains as before in the hot methylen-blue solution for not more than half a minute, and washes out as before with 96 per cent. alcohol. The sections are then stained for fifteen to thirty minutes in a 0·1 per cent. solution of fuchsin in 96 per cent. alcohol, washed out for a minute, until no more red colour comes away, in alcohol, cleared in clove oil, and mounted. Nerve-cells blue-red, their nuclei being unstained; nuclei of connective tissue and of vessels, brilliant red.

This distinction is not obtained in embryonic tissues.

Nuclei of connective tissue and vessels may also be brought out by staining for a few minutes in 1 per cent. aqueous solution of eosin, followed by a few minutes in warm 0·1 per cent. aqueous solution of dahlia, dehydration, and mounting (the nuclei blue, all else red). Or 1 per cent. aqueous solution of nigrosin may be taken instead of the eosin, and 0·1 per cent. alcoholic solution of fuchsin (half an hour) instead of the dahlia (nuclei red, all else grey-blue).

754. *Hæmatoxylin* may be used as a general stain. Bevan Lewis recommends the formula of Kleinenberg, or a formula that he attributes to Minot, which is essentially the same as Böhmer's. BERNHEIMER'S formula, which is quoted in regard to the retina, § 683, is practically the same thing.

MALLORY'S **Phospho-molybdic Acid Hæmatoxylin Stain** has been given in § 195.

SCHIEFFERDECKER and VOBIS find that celloidin sections of material hardened in Müller stain well in it.

755. HAUG (*Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 153) gives the following modification of WEIGERT'S process, as being more convenient to carry out, and serving to demonstrate all the elements of spinal cord with equal clearness. Small segments of fresh cord are put into saturated aqueous solution of neutral acetate of copper. After two days (or, for large specimens, longer) they are put for a day or a day and a half into a 5 per cent. or even saturated solution of bichromate of potash. They are next rinsed with water and put for thirty-six to forty-eight hours into 70 per cent. alcohol in the dark. They are then treated for the same time with absolute alcohol, also in the

dark; then imbedded, either in paraffin or celloidin, and cut. The sections, after removal of the paraffin (if that have been used), are treated with alcohol, and then with water. They are stained for a quarter or half an hour in a well-ripened mixture of 1 part of hæmatoxylin, 30 parts of alcohol, 1 part of ammonia alum, and 300 parts of water (or they may be treated for a quarter of an hour to an hour with saturated solution of carbonate of lithia, and then for an hour, in total darkness, with simple saturated aqueous solution of hæmatoxylin). After staining to a deep black they are rinsed in water and differentiated until they turn red (a quarter of an hour at most), either in 70 per cent. alcohol acidified with 0·5 to 1 per cent. of hydrochloric acid or in a solution containing 0·5 part of oxalic acid, 0·1 to 0·25 of hyposulphite of soda, and 100 of water. They are then washed with pure water until they turn blue or bluish grey; after which they may either be mounted, or first counterstained by momentary immersion in a strong solution of neutral carmine. If desired, sections after staining may be differentiated in Weigert's borax-ferricyanide liquid, instead of the liquids here given.

756. *Alizarin* has been recommended by Benczur (see Thanhoffer's *Das Mikroskop;* or *Zeit. f. wiss. Mik.*, 1884, p. 97). It is directed that sections be stained for twenty-four hours in a concentrated solution of alizarin in alcohol.

Anilin blue alone is useful for the demonstration of ganglion-cell processes (see Zuppinger, in *Arch. f. mik. Anat.*, 1874, p. 255).

757. *Anilin blue-black* was first recommended by Sankey (*Quart. Journ. Mic. Sci.*, 1876, p. 69). He stained in a 0·5 per cent. solution, and, in order to obtain a differential stain, washed out for twenty to thirty minutes in solution of chloral hydrate. Bevan Lewis (*Human Brain*, p. 125) considers this to be one of the most valuable stains for nervous centres. He stains sections for an hour in 0·25 per cent. aqueous solution, and clears and mounts (in the case of brain or cord sections); for the cortex of the cerebellum he washes out for twenty to thirty minutes in 2 per cent. chloral solution. Sankey and Stirling have also used anilin blue-black in a much weaker solution, which Bevan Lewis does not recommend. Vejas, however (*Arch. f. Psychiatrie*, xvi, p. 200), obtained good results by staining from eighteen to twenty-four hours in a solution of 1 in 3000.

Gierke (*Zeit. f. wiss. Mik.*, 1884, p. 379) was not able to obtain good results with anilin black procured in Germany, and finds that the treatment with chloral is injurious to the preservation of the tissues. Martinotti (*ibid.*, p. 478) comes to the same conclusion.

Luys (*Gaz. méd. de Paris*, 1876, p. 346) greatly recommends

the anilin colour known as *Noir Colin*. He stains for three to four minutes in a 0·1 per cent. solution.

Jelgersma (*Zeit. f. wiss. Mik.*, 1886, p. 39) finds that anilin blue-black gives excellent results, *provided that the English preparation of the colour be alone employed.* He makes solutions of 1 : 100, 1 : 800, and 1 : 2000, of which the first stains sections in a quarter of an hour, the second in five hours, the third in twelve hours. The stain takes effect on ganglion-cells and their processes, and on axis-cylinders, but does not demonstrate neuroglia or connective tissue.

Schmaus (*Münch. med. Wochenschr.*, No. 8, 1891, p. 147; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 230) recommends English blue-black in 0·25 per cent. solution in 50 per cent. alcohol, with the addition of a little picric acid; sections to be stained for an hour. The addition of picric acid has the advantage of leaving celloidin almost colourless, whilst it stains strongly in pure aqueous solutions of blue-black.

Martinotti (l. c., 1884, p. 478) finds that *picro-nigrosin* gives very good results, especially for pathological objects. He stains for two or three hours or days in a saturated solution of nigrosin (§ 125) in saturated solution of picric acid in alcohol, and washes out in a mixture of 1 part of formic acid with 2 parts of alcohol until the grey matter appears clearly differentiated from the white to the naked eye.

758. Kaiser (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 471) advises, for celloidin sections of spinal cord, naphthylamin brown (obtainable from Grübler). Sections are stained for a few hours in a solution containing 1 part of naphthylamin brown, 200 parts of water, and 100 parts of alcohol, washed with alcohol, cleared with origanum oil, and mounted. Chromophilous ganglion-cells, dark brown; chromophobous cells, light on a dark ground. Black-ground illumination is recommended. The stain has the advantage of not over-staining, and not staining celloidin to an injurious extent.

759. The Ehrlich-Biondi mixture of methyl green, orange, and Säurefuchsin, which has been given in the chapter on "Combination Stains," § 259, is a good stain for nerve-centres, especially (Dr. Lindsay-Johnson informs me) if there be added to it about one-third of 20 per cent. (saturated) solution of nigrosin.

760. Golgi's **Sublimate Method.**—Last, but not least, as a general stain we have the *sublimate method of* Golgi (*Archivio*

per le Scienze Mediche, 1878, p. 3). This method, which may be said to be in principle identical with the bichromate of potash and silver nitrate method of the author, consists, like the latter, of two processes: 1, hardening in bichromate; 2, treatment with bichloride of mercury.

For hardening, use either a solution progressively raised in concentration from 1 per cent. to 2½ per cent., or Müller's solution. Take small pieces of tissue (not more than 1 to 2 c.c.), large quantities of liquid, and change the latter frequently, so as to have it always clear. Fifteen to twenty days' immersion will suffice, but twenty to thirty should be preferred.

The tissues are then passed direct from the bichromate into the bichloride of mercury. The solutions of the latter employed by Golgi varied from 0·25 per cent. to 0·50 per cent.: he cannot say which strength is to be preferred. The immersion in the bichloride must be much longer than the immersion in the nitrate of silver bath of that process: for the latter, twenty-four to forty-eight hours suffice; but in the bichloride an immersion of eight to ten days is necessary in order to obtain a complete reaction through the whole thickness of the tissues. During the bath the bichromate will diffuse out from the tissues into the bichloride, which must be changed every day; at the end of the reaction the preparations will be found decolourised, and offering the aspect of fresh tissue. They may be left in the bichloride for any time.

In *Rendiconti R. Ist. Lombardo di Sci. Milano*, 2, xxiv, 1891, pp. 594, 656 (see *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 388) Golgi says that for the study of the diffuse nervous reticulum of the central nervous system the best results are obtained by keeping the preparations in 1 per cent. sublimate for a very long time, two years being not too much in some cases.

Before mounting, the sections that have been cut must be repeatedly washed with water (if it be wished to mount them permanently), otherwise they will be spoilt by the formation of a black precipitate. In the last place quoted Golgi says that after washing they may be toned by putting them for a few minutes into a photographic fixing-and-toning bath, after which it is well to wash them again, and stain them with some acid carmine solution. Mount in balsam or glycerin; the latter seems the better preservative medium.

The result of this process is not a true stain, but an "apparently black reaction," the tissues appearing black by transmitted light, *white* by reflected light. Golgi thinks that there is formed in the tissue elements a precipitate of some substance that renders them *opaque*. The elements acted on are (1) the ganglion-cells, with all their processes and ramifications of the processes. These are made more evident than by any other process except the bichromate and silver-nitrate process. An advantage of the mercury process is that it demonstrates nuclei, which is not the case with the silver process. (2) Connective-tissue corpuscles in their characteristic radiate form. But the reaction in this case is far less precise and complete than that obtained by the silver process. (3) The blood-vessels, and particularly their muscular fibre cells.

The method gives *good* results only with the cortex of the cerebral convolutions, hardly any results at all with the spinal cord, and very scanty results with the cerebellum. And, on the whole, the method shows nothing more than can be demonstrated by the silver-nitrate method, but it is superior to it as regards two points: the reaction can always be obtained with perfect *certainty* in a certain time, and the preparations can be perfectly preserved by the usual methods.

Tal (*Gazz. degli Ospitali*, 1886, No. 68) finds that if sections made by this process be treated with solution of sodium sulphide, a much darker stain is obtained. Sections may then advantageously be double-stained with Magdala red.

Golgi's method may be combined with Weigert's nerve stain (see Pal, *Wien. med. Jahrb.*, 1886; *Zeit. f. wiss. Mik.*, v, 1, 1887, p. 93).

761. Cox (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 16; *Journ. Roy. Mic. Soc.*, 1891, p. 420) finds the sublimate and bichromate may be used *together*, and give a uniform impregnation. He used a fluid consisting of 20 parts 5 per cent. bichromate, 20 parts 5 per cent. sublimate, 16 parts 5 per cent. simple chromate of potash, and 30 to 40 parts of water. The mixture should be as little acid as possible. The duration of the impregnation is from two to three months. There is considerable difficulty in preserving sections, which must be made with a freezing microtome, alcohol being avoided, treated with solution of sodium carbonate, and mounted without a cover.

762. Magini (*Boll. Accad. Med. di Roma*, 1886; *Zeit. f. wiss. Mik.*, 1888, p. 87) recommends a development of Golgi's process in which zinc chloride is used in place of sublimate. Portions of tissue of 2 to 3 cm. cube

are hardened for at least two or three months in Müller's solution. They are well washed with distilled water, and brought into a 0·5 to 1 per cent. solution of chloride of zinc. This is changed for fresh every day for seven to ten days (until it does not become yellower than bichromate solution). Sections are then made, washed quickly with alcohol, imperfectly cleared (see § 779) with kreasote, and mounted in dammar. This process is said to demonstrate better than Golgi's the finer structure of ganglion-cells and their processes.

763. Flechsig (*Arch. f. Anat. u. Phys.*, Physiol. Abth., 1889, p. 537 ; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 71) has a process for combining Golgi's impregnation with v. Branca's redwood stain (*ante*, § 694) and a gold chloride impregnation, which seems to me too complicated to be trustworthy.

764. Monti (*Atti d. R. Accad. dei Lincei Roma*, Rendic., v, 1889, 1 sem., p. 705 ; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 72) describes a brown impregnation brought about by the combined action of bichromate of potash and sulphate of copper. The method does not appear to have been yet brought to a practical form.

β. *Special Stains.*

765. The most important of the methods for the study of nerve-tracts, plexuses, and the finer relations of nerve-fibrils and ganglion-cell processes are those that have already been given in the last Chapter, together with some of those given in this Chapter, and classed as general stains.

As *myelin stains* we have the processes of Weigert, §§ 688 and 703, and the modifications of the same by Max Flesch, § 689 ; Benda, 690 ; Pal, § 692 (very important) ; Schäfer, § 693 ; Kultschizky, § 697 ; Wolters, §§ 699, 700, and others ; and as processes less exclusively directed to the demonstration of the myelin element, and claiming more particularly to serve for the demonstration of *axis-cylinders* and *cell processes*, the chromate of silver impregnation methods of Golgi, §§ 704 to 706 (of the highest importance, and of the widest applicability), with the modifications of the same by Sehrwald, § 708 ; Greppin, § 710 ; Obregia, § 711 ; Ramón y Cajal, § 714, and others, amongst which I would particularly call attention to the details of the process as carried out by Ramón y Cajal ; and amongst the others later described that of Wolters, § 726, and the iodide of palladium method of Paladino, § 727.

There remain to be mentioned some other methods, based for the most part on somewhat different principles.

766. The **methylen blue** impregnation method has been described in Chap. IX, and its great importance has been insisted on.

767. The Gold Method is one of the most important methods for the study of the finer nerve-fibrils of the spinal cord. The instructions given by GERLACH (*Stricker's Handb.*, p. 678) have been already quoted, *ante*, § 228, p. 148.

BOLL (*Arch. f. Psych. u. Nervenk.*, iv, p. 42 ; GIERKE, *Zeit. f. wiss. Mik.*, 1884, p. 403) makes the following observations on Gerlach's process :—The hardening in bichromate ought not to be prolonged further than is absolutely necessary, for after eight days the elective susceptibility of the tissues for gold impregnation begins to diminish, and after fifteen days has almost disappeared. The knife should not be wetted with alcohol; the tissues should never be allowed to come into contact with alcohol. Only a small quantity of gold solution, relatively to the volume of the sections, should be taken. The impregnation should last for twelve hours.

Gierke (l. c.) says, "Gerlach's gold preparations have never had their equal for the demonstration of very fine nerve-fibrils. Weigert's Säurefuchsin preparations are the only ones that can be compared with them in this respect."

SCHIEFFERDECKER (*Arch. f. mik. Anat.*, 1874, p. 472) also recommends gold chloride for demonstrating fine networks in *transverse* sections. Strength 1 : 5000 or 10,000. Time about one to three hours. After washing in water the sections are put for twenty-four hours into acetic acid of $\frac{1}{2}$ to 1 per cent., then mounted in balsam.

According to the same author, *chloride of palladium* may advantageously be used for the demonstration of the *longitudinal* fibres, and (of course, therefore) for staining longitudinal sections. A solution of 1 : 10,000 strength is taken ; the sections remain in it till light brown (about three to five hours). For some further methods of the same author see l.c., 1878, p. 38.

MERKEL'S chloride of palladium and carmine method has been given above, § 746.

768. FREUD'S *gold method* (*Arch. f. Anat. u. Phys.*, 1884, p. 453) may be used for controlling the results obtained by Weigert's process, and for obtaining an impregnation of axis-

cylinders. This method is based on that of FLECHSIG (see § 228), which I suppress, as it gives less certain results. Freud proceeds as follows:—Sections of material hardened in solution of Erlicki (which may be followed by alcohol without hindrance to the impregnation) are washed with water, and put for three to five hours into 1 per cent. solution of gold chloride. After again washing with water they are treated for three minutes with a solution of 1 part of caustic soda in 5 or 6 of water. They are then drained (not washed) and brought into 10 or 12 per cent. solution of iodide of potassium. After from five to fifteen minutes therein they are washed with water, dehydrated, and mounted in balsam. (In the case of objects that stain easily it is useful to dilute the gold solution with a volume or two of alcohol; the stain is more highly selective.)

BECKWITH (*Journ. Roy. Mic. Soc.*, 1885, p. 894) modifies this process by intercalating a treatment for thirty minutes with 10 per cent. solution of carbonate of potash between the soda and the iodide.

769. ZIEHEN'S *gold and sublimate method* (*Neurol. Centralb.*, x, 1891, No. 3, p. 65; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 385) gives the following derivative of Golgi's sublimate method as an improvement on it:—Small pieces of fresh material are thrown into a large quantity of a mixture of 1 per cent. sublimate solution and 1 per cent. chloride of gold solution in equal parts. They remain therein for at least three weeks, preferably for several months (up to five), by which time they will have become of a metallic red-brown colour. They are gummed on cork and sectioned without imbedding. The sections are treated either with solution of Lugol (§ 66) diluted with four volumes of water, or with dilute tincture of iodine, until duly differentiated, which will require more or less time according to the thickness of the sections. They are then washed and mounted in balsam. The result is a bluish-grey impregnation; both medullated and non-medullated nerve-fibres are stained, also nerve- and glia-cells and their processes.

770. UPSON'S **Methods** (MERCIER, in *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 474: I pass over the older methods given in *Neurol. Centralb.*, 1888, p. 319; and *Zeit. f. wiss. Mik.*, 1888, p. 525).

The Gold and Iron Method.

Material must be very carefully hardened for from four to six months in bichromate of potash (*not* solution of Müller, which does not allow of the same precision of stain). A 1 per cent. solution should be taken at first, and should at first be frequently changed. Only after some weeks should the strength be gradually increased up to 2 or 2·5 per cent. All the hardening should be done in the dark. Over-hardened material is not available. The surface of section of a properly hardened piece of material should show microscopically no marked difference of colour between the white and the grey matter. If the white substance appear very dark, almost black, and the grey substance whitish grey, good results will probably not be obtained; a medullary stain will probably result, whereas the method ought to give an axis-cylinder and cell-process stain.

After hardening, the specimens are washed with water, and put for two or three days into 50 per cent. alcohol, changed as often as necessary, and then into 95 per cent. alcohol, in which they should remain until they show a decidedly green coloration (two to four weeks), the alcohol being changed as often as precipitates occur. Sections are made either under water or by the celloidin process (in the former case they should not be allowed to remain in contact with the water longer than is absolutely necessary, and should be put back into alcohol for two or three days before passing to the next stage).

The next stage consists in a bath of from one to two hours' duration in 1 per cent. solution of chloride of gold acidified with 2 per cent. of hydrochloric acid. After this the sections are rinsed with water, and treated for half a minute with a freshly prepared solution of ferricyanide of potassium in 10 per cent. potash solution (a lump of ferricyanide not half so big as a pea to 5 c.c. of the potash solution). They are then washed for half a minute more in pure 10 per cent. potash solution, and after that for some time in distilled water.

The following reducing mixture (which must be freshly made up at the very instant of using it for each section) is now to be prepared:

Acidum Sulfurosum . . . . 5 c.c.
Tinctura Iodi (3 per cent. strength) . 10—15 drops.
Mix, and add—
Liquor Ferri Chloridi . . . . 1 drop.

You must mix quickly in a graduated glass. The section should now be brought on a piece of filter-paper into a watch-glass, and the reducing mixture should be poured over it evenly at one pour, just as developing solution is poured on to a photographic negative. The section should remain in this bath until it is of a fine rosy red tint, not a second longer, or it will become reddish black and useless. As soon as the rose-red colour appears the section is removed into distilled water. The water is changed once, the section is placed on a slide aad brought into absolute alcohol, and after five to fifteen minutes therein into clove oil and mounted in balsam. The sections must be kept in the dark.

(The treatment with ferricyanide of potassium sometimes gives rise to the formation later on of precipitates of Berlin blue in the tissues; and as this treatment is not essential it may be omitted, the sections being simply treated with pure potash solution as directed.)

771. Upson's Methods—the Gold and Vanadium Method.—Sections (made as before) are placed in the following solution:

1 per cent. chloride of gold solution . . . . . 5 c.c.
Saturated solution of vanadate of ammonium . . . . . 10 drops.
Hydrochloric acid . . . . 3 „

After two hours therein they are washed with distilled water, and put for half to one minute into a mixture of—

10 per cent. solution of caustic potash . 5 „ .
10 per cent. solution of permanganate of potash . . . . . 10 drops.
Vanadate of ammonium . . . a trace.

They are rinsed in distilled water, and treated until they become red, as in method *a*, with the following freshly prepared reducing fluid:

a. 3 per cent. tincture of iodine, to which has been added chloride of tin until of

a white or yellowish tint (may be kept in stock)	15 drops.
b. Distilled water	3 c.c.
c. Saturated solution of phosphate of iron in distilled water (the usual pharmaceutical solution)	3—5 drops.
d. Sulphurous acid	3 c.c.

Mix *a*, *b*, and *c*, and add *d*, which will cause a voluminous precipitate, at which instant the mixture is to be poured over the section, or the section brought into it, as in method *a*, the remaining treatment being exactly as there described.

The principle of the two methods is the decoloration of the impregnated elements (especially glia-cells) up to a certain point by means of the potash, or potash and ferricyanide, or the vanadate and permanganate. It is important to take care that this decoloration be not overdone, as if carried too far the axis-cylinders and ganglion-cells will be decolourised.

772. Weigert's *Säurefuchsin* method mentioned above appears to be definitively superseded by his hæmatoxylin method. See, however, *Centralb. f. d. med. Wiss.*, 1882, pp. 753, 772; *Fortschr. d. Med.*, 1884, Nos. 4 and 6; *Zeit. f. wiss. Mik.*, 1884, pp. 123, 290.

773. Sahli (*Zeit. f. wiss. Mik.*, 1885, p. 1) gives the following method:—Sections of tissue hardened in bichromate to the degree required for Weigert's hæmatoxylin process are washed for not more than five or ten minutes in water, and stained for several hours, until they are of a dark blue colour, in concentrated aqueous solution of methylen blue. They are then rinsed with water, and stained for five minutes in saturated aqueous solution of Säurefuchsin. If now they be rinsed with alcohol and brought into a liberal quantity of water, the stain becomes differentiated, axis-cylinders being shown coloured red and the myelin sheaths blue. If, instead of rinsing with pure alcohol, alcohol containing from 0·1 to 1 per cent. of caustic potash be taken, the stain differentiated in water, and the sections cleared with cedar oil and mounted in balsam dissolved in cedar oil, still finer images are obtained. Axis-cylinders are red as before, but the myelin sheaths are blue in some places, red in others. Sahli thinks that this reaction points to some difference of kind in the nerve-tubes that exhibit it.

The same author (l. c., p. 50) also gives a method for obtaining a specific stain of nerve-tubes by means of *methylen blue alone*. Sections of material hardened as before are stained for a few minutes or hours in the following liquid:

Water	40 parts.
Saturated aqueous solution of methylen blue .	24 „
5 per cent. solution of borax	16 „

(Mix, let stand a day, and filter.)

The sections are then washed either in water or alcohol until the grey matter can be clearly distinguished from the white, are cleared with cedar oil, and mounted in balsam. Nerve-tubes are stained blue, ganglion-cells greenish, nuclei of neuroglia blue. Micrococci are stained, if any be present in the tissues. The preparations are not perfectly permanent.

774. *Safranin followed by methylen blue* gives a very special stain of spinal cord. The method is due to ADAMKIEWICS (*Sitzb. k. Acad. Wiss. Wien, Math. Naturw. Kl.*, 1884, p. 245; *Zeit. f. wiss. Mik.*, 1884, p. 587). Sections are washed first with water, then in water acidified with a little nitric acid, and stained in concentrated solution of safranin. They are then treated with alcohol and clove oil till no more colour comes away, and are brought back again into water, washed in water acidified with acetic acid, stained in methylen blue, and cleared as before. The process is said to demonstrate the existence of "chromoleptic zones" which surround the grey matter. Myelin ("erythrophilous substance" of Adamkiewics) is red, nuclei of nerves, of neuroglia, and of vessels violet. The erythrophilous substance of pathological nerve-tubes does not take the stain, so that the method is valuable for the study of degenerative changes.

775. NIKIFOROW (*Zeit. f. wiss. Mik.*, v, 3, 1888, p. 338) modifies the foregoing method as follows:—The tissue is to be hardened in a chrome salt. It is not to be washed in water afterwards. Sections are brought direct from alcohol into the safranin stain, either concentrated aqueous, or Babes's solution, or a solution in 5 per cent. carbolic acid. Stain for twenty-four hours. Bring the sections into alcohol until the colour is sufficiently extracted for the grey substance to be distinguished from the white. Then put them into a weak (0·1 to 0·2 per cent.) solution of chloride of gold or platinum or any metallic salt. They are to be left in this until the grey substance just begins to get a violet tone (not longer). They are then well washed in water, and put into alcohol until the rosy violet of the grey substance becomes distinctly marked off from the red of the white substance. Clear with clove oil, wash out the clove oil thoroughly with xylol (this is absolutely necessary for the permanence of the preparations), and mount in balsam. Nikiforow says that this method gives much sharper and more certain results than that of Adamkiewics.

776. *Purpurin*, according to DUVAL, has a special action on nerve-tissue (especially spinal cord) that has been hardened in bichromate of ammonia. Nerve-cells and their processes, axis-cylinders and connective-tissue fibrils, remain unstained, whilst the nuclei of connective tissue and of capillaries stain red (see § 201).

Mounting.

777. General Mounting Methods.—Under this head it is to be noted that it is often advisable to fix sections to the slide before applying balsam, or other medium, and a cover. For celloidin sections you may use one of the usual methods described in Chap. XVII, or for large sections the gelatin method of Fol, or Minot's shellac method. This is as follows:

—You arrange the sections on the slide, and cover them with a layer of 10 to 12 per cent. solution of shellac, then warm to 30° or 40° C., until the shellac appears dry, clear with clove oil, and mount in balsam (*Zeit. f. wiss. Mik.*, 1886, p. 175). This process is applicable either to paraffin or collodion sections.

778. Serial Section Mounting.—Besides the ordinary methods for mounting small sections, there are certain processes specially adapted to the mounting of very voluminous sections. One of these is GIACOMINI's collodion-gelatin method, which is certainly a valuable one. But it is hardly more than a macroscopic method principally adapted for the study of the coarser topography of the brain, and as such may be passed over here, though I would willingly include it did our shortening space allow. Descriptions of the process are to be found in *Gazzetta delle Cliniche*, Nov., 1885; *Zeit. f. wiss. Mik.*, 1885, p. 531; and *Traité des Méth. techn.*, LEE et HENNEGUY, p. 392.

A further reason for passing over this process is that the same end may be attained, in many cases more effectually, by the beautiful methods of WEIGERT (§ 340), STRASSER (§ 326), and APÁTHY (§§ 338, 339), which see.

A useful method for small sections is that of OSBORN, who arranges his sections, wet with alcohol, in order on the slide, covers them with a cigarette paper, and treats them with the clearing agent through the paper.

For DEECKE's method see above, § 743.

Other Methods for Nervous Centres.

779. The Half-clearing Method.—Since the days of Lockhart Clarke, to whom the method is due, most observers have been struck by the wealth of minute structure revealed by half-cleared sections. Detail that is invisible in perfectly cleared sections stands out in sculptural relief during the period in which the tissues are only about half cleared. It is of course most important to be able to preserve preparations permanently in this state.

MERKEL (*Arch. f. mik. Anat.*, 1877, p. 622) gives the following instructions:—Sections are dehydrated with alcohol of about 94 per cent. (in which they must remain for at least ten minutes), and then cleared with xylol, in which they are examined.

Certain elements of the tissues retain more obstinately than others the small quantity of water that they bring with them out of the 94 per cent. alcohol. Now, as xylol is absolutely immiscible with water, it can exercise no clearing action on these, and they stand out boldly in the picture by virtue of the difference between the index of refraction of their contained water and that of the xylol. The axis-cylinders are at first all that is visible; after a time the ganglion-cells appear with their processes. Nuclei, vessels, and whatever else may be in the preparation are totally invisible. Merkel thinks this method superior to all others for the study of the distribution of nerve-fibres. The preparations are not permanent, though they may be kept for some weeks by mounting them in Canada balsam. When a preparation (either in xylol or balsam) has become so transparent as to be of no further

use (which will always eventually happen), it may be reprepared by putting it back into the alcohol, and thence again into the xylol.

BEVAN LEWIS (*The Human Brain*, p. 122) proceeds as follows:—A section is placed, saturated with spirit, on a slide. When the spirit has nearly all evaporated, a drop of oil of anise is allowed to flow *over* the section (not to float it up), and the clearing is watched on the stage of the microscope; then, just when the desired image is arrived at, a drop of balsam is allowed to run over the section, and a cover put on.

Instead of oil of anise, Lewis has frequently employed glycerin followed by mounting in glycerin jelly, with the same results.

BYROM BRAMWELL (*Edinb. Med. Journ.*, Oct., 1886) also recommends the process. He uses clove oil.

780. GIACOMINI'S "Dry" Process for Preserving Brains (*Arch. per le Scienze Mediche*, 1878, p. 11).—Although this is in intention a *macroscopic* method, it appears worth while, both on account of its thorough success and on account of its suggestiveness, to give a description of it here.

The object is to make "dry" preparations of the encephalon; by which is meant preparations that can be permanently preserved *in the air*. The methods hitherto employed were not successful because they consisted in making preparations that were "dry" in the literal sense of the word—that is, deprived of their natural water; and since brain-substance contains 88 per cent. of water, such preparations could not of course be obtained without so great an amount of shrinkage as to most seriously diminish the scientific value of the result. The principle of Giacomini's method is, on the contrary, to *retain* the natural water of the tissues, or an equivalent for it, by means of impregnation with a hygroscopic substance,—glycerin.

The process consists of two divisions:—1, hardening; 2, impregnation with glycerin.

1. For hardening may be used zinc chloride, bichromate of potash, chromic acid, nitric acid, or alcohol.

Chloride of zinc gives the best results. Perfectly fresh brain is put into a saturated aqueous solution of the salt (if there be reason to fear that the tissues are somewhat softened through having been left too long after the death of the subject, it is well first to inject 600 grms. of the solution through the internal carotid arteries). After forty-eight hours' immersion (during which time the floating brain must be turned over three or four times, so that all parts of it may duly come into contact with the liquid) the surface of the brain will have attained a consistency that will allow of the removal of the arachnoid and pia mater. The meninges having been removed, the encephalon is put back into the solution for two or three days more, during which time it will be seen that, increasing in specific gravity, it tends towards the bottom of the vessel containing it. When this is seen to happen it must be removed into commercial alcohol, as if allowed to remain longer in the chloride of zinc solution it would take up too much water.

In the alcohol it may remain for an indefinite time, or it may be removed if desired after ten or twelve days. (During the alcohol-bath it must be frequently turned over in order that no malformation may arise from continuance of pressure on the same part.)

It is then removed into glycerin (either pure or with 1 per cent. of carbolic acid). It floats at first, but gradually sinks as the alcohol evaporates. As soon as it has sunk just below the surface it may be removed and exposed to the air.

It is set aside to "evaporate" in a convenient place for a few days. As soon as the surface has become dry, it is varnished with india rubber or (better) with marine glue varnish diluted with a little alcohol. This completes the process.

If it be desired to make dissected preparations, the necessary dissection should be made on removing the encephalon from the alcohol before putting into glycerin.

Bichromate of potash may be used for hardening in solutions gradually increasing in concentration from 2 to 4 per cent. The liquid must be frequently changed; the immersion must be of not less than a month's duration. Six to eight days will suffice for the alcohol-bath, or this may be altogether omitted.

Nitric acid is used in solutions of from 10 to 12 per cent. for twelve to fifteen days. (Encephala float in this liquid, and must therefore be frequently turned over. It is this reagent that gives the *toughest* preparations.)

Concerning the value of the process, Golgi (from whose abstract I take the foregoing account) states that after a series of experiments he is able to affirm that for preservation of the volume, the colour, the finer relations of the parts, and the physiognomy proper to the organ, the process is far superior to any hitherto known. I am able to add that I saw specimens of Giacomini's preparations at the Milan International Exhibition of 1881, and think it would be hard to over-praise their beauty of aspect.

It should be added that histological detail is preserved to a remarkable extent by this process, and that excellent sections may be cut at any time from the hardened brains. And as the preparations take up as little room as possible, there seems no reason why the process should not be generally adopted in medical schools, lunatic asylums, and similar institutions.

The method may also be applied, with most perfect success, to the preservation of small animals entire, such as Batrachia, Reptilia. It is well to inject them with the zinc solution.

Another "dry" method has been given by Max Flesch (*Mt. Naturf. Ges. Bern.*, 1887, p. xiii). He hardens in alcohol, and then brings the brains through Calberla's mixture (§ 405) into glycerin containing (1 part to 3000 of) sublimate. He does not appear to varnish his preparations. See *Journ. Roy. Mic. Soc.*, 1888, p. 507.

781. Dissociation Methods.—Maceration of nervous tissue is a method that rendered service years ago for the isolation of ganglion-cells with their processes, &c. These details can, however, now be studied *in situ* by means of the excellent methods of special staining or impregnation that have been given in the foregoing pages, so that the maceration methods can hardly claim now to be useful for purposes of research, and may at most claim to serve as means of demonstrating to beginners the general characters of ganglion-cell processes. See, however, the following:

Stilling's method (*Arch. f. mik. Anat.*, 1880, p. 471; *Bau. d. nervösen Centralorgane*, 1882).

Carrière's (*Arch. f. mik. Anat.*, 1877, p. 126).

Schiefferdecker's (*ibid.*, 1878, p. 38; also Behrens, Kossel, und Schiefferdecker, *Das Mikroskop*, Bd. ii, p. 227).

Freeborn's (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 298).

782. Bevan Lewis's **Compression Method for Fresh Tissue** (*Mon. Mic. Journ.*, 1876, p. 106; *Human Brain*, p. 146) is as follows:—Vertical sections, as thin as possible, are made from a piece of a convolution of brain from which the membranes have been removed. The sections are made by free hand by means of a section-knife flooded with spirit. The sections are got on to a slide and treated with Müller's solution for a few seconds. A cover is then put on and steadily pressed down so as to flatten out the sections into an almost transparent film. The slide is then rinsed in water and placed for thirty to forty seconds in a bath of methylated spirit. It is then removed, one edge of the cover-glass steadied with the finger, the blade of a penknife inserted under the opposite edge, and the cover gently lifted. The section, which remains adherent either to the slide or to the cover, is washed with water, stained in any way that may be desired, dehydrated, and mounted in balsam.

For staining the cells of the cortex, Lewis prefers a 1 per cent. solution of anilin black.

He prefers to dehydrate by drying under a bell-glass in the presence of concentrated sulphuric acid. Clear with chloroform in preference to clove oil. Lewis finds that this process results in far less rupture and tearing of nerve-cells and processes than would be imagined; he considers that "the result is equivalent to the most delicate teasing of tissue, the processes being gradually unravelled from their dense networks, and the structural elements universally displayed to the best advantage."

v. Thanhoffer (*Zeit. f. wiss Mik.*, iv, 4, 1887, p. 467) describes a similar process.

783. Neuroglia (Gierke, *Arch. f. mik. Anat.*, 1885, p. 444). —Maceration in the usual media, chromic solutions, iodised serum, &c., but especially in the liquid of Landois (§ 532). For sections, harden for six to ten weeks in bichromate of ammonia, first of 1·5 per cent., and gradually raised to 3 per cent. Avoid paraffin for imbedding, and imbed in celloidin or cut without imbedding. Stain with ammonia-carmine, or picro-carminate of soda, or alum-carmine, or Heidenhain's hæmatoxylin. Other details in this valuble paper.

784. Myelon of Reptilia and Amphibia.—Besides the usual methods which will suggest themselves as being applicable to this class of objects, see Mason's methods, *supra*, § 742; Schmidt, *Zeit. f. wiss. Mik.*, 1885,

p. 389 ; v. LENHOSSEK, *Arch. f. mik. Anat.*, 1886, p. 379 (minute directions for dissecting out the spinal ganglia of *Rana*); KORYBUTT-DASZKIEWICS, *ibid.*, 1889, p. 51 ; or *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 203; OSBORN, *Journ. Roy. Mic. Soc.*, 1885, p. 536 ; or WHITMAN'S *Methods*, p. 195.

785. APÁTHY'S **double stain** for differentiating nervous tissue and connective tissue (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 170) consists of staining with Heidenhain's hæmatoxylin, and after-staining with very weak alum hæmatoxylin.

MARCHI'S **Method for Degenerate Medullated Nerve-fibres.**—See § 720A.

CHAPTER XXXI.

SOME OTHER HISTOLOGICAL METHODS.

Connective Tissues.

786. Connective Tissue.—S. Mayer (*Sitzb. k. Akad. Wiss.*, lxxxv, 1882, p. 69) recommends, for staining *fresh* tissue, a solution of 1 gramme of "Violet B" (Bindschedler and Busch, Bâle) in 300 c.c. of 0·5 per cent. salt solution. In this liquid connective-tissue cells stain rapidly and energetically. Elastic fibres and smooth muscle also stain, but of different tints.

Freeborn (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 305) recommends (for sections) picro-nigrosin, made by mixing 5 c.c. of 1 per cent. aqueous solution of nigrosin with 45 c.c. of aqueous solution of picric acid. Stain for three to five minutes, wash with water, and mount in balsam. Connective-tissue fibres bright blue, nuclei blackish, all the rest greenish yellow. Sections may be after-stained for five or six minutes in a mixture of 1 c.c. of saturated alcoholic solution of eosin and 49 c.c. of alcohol. The results are as before, except that the yellow colour is replaced by red.

For Ranvier's method of artificial œdemata for the study of areolar tissue, see his *Traité*, p. 329.

For Flemming's observations on the development of connective-tissue fibrils, see *Festschr. R. Virchow gewidmet*, &c., 1, 1891, p. 215; *Zeit. f. wiss. Mik*, ix, 2, 1892, p. 225.

For Pianese's very complicated double stain with carmine and picro-nigrosin, see *Journ. Roy. Mic. Soc.*, 1892, p. 292.

Note also the Ehrlich-Biondi stain, § 259.

787. Fat.—Dekhuysen (see Flemming, in *Zeit. f. wiss. Mik.*, 1889, pp. 39, 178) has discovered that fat that has been stained black by treatment with chromo-aceto-osmic acid (*not* with pure osmium) is dissolved in the course of a few hours in turpentine. It is dissolved also in xylol, ether, and kreasote. Flemming finds that very good demonstration preparations may be made by treating fatty tissue with chromo-aceto-osmic acid, staining with safranin or gentian, and then treating for a few hours with turpentine until all the fat is dissolved. The optical hindrance caused by the high

refraction of the fat being thus eliminated, nuclei and cytoplasm may be studied to far greater advantage than in the usual preparations.

788. Granule-cells ("Plasmazellen" and "Mastzellen").—In 1874 there were described by WALDEYER (*Arch. f. mik. Anat.*, Bd. xi) certain special cells existing between the bundles of connective tissue, besides the flat cells, and lymphatic and fat cells. They are large round cells containing large granules; Waldeyer called them *plasma cells* ("Plasmazellen"). Later on, EHRLICH (*ibid.*, xiii, 1877) distinguished in the same tissue and in other places certain cells containing large granules, which have a superficial resemblance to Waldeyer's plasma cells, but which differ from them in staining reaction (*Verhandl. Berl. Physiol. Ges.*, January 17, 1879; *Reichert u. Du Bois Reymond's Arch.*, 1879, p. 166). Ehrlich named these *food cells* ("Mastzellen"), intending to express thereby the opinion that these cells are derived from fixed connective-tissue cells by a transformation brought about by exalted nutrition.

If (KORYBUTT-DASZKIEWICZ, *Arch. f. Mik. Anat.*, xv, 1878, p. 7) frogs be kept for two months (in snmmer) without food, then placed in a reservoir of running water and well fed for four weeks, "plasma-cells" will be found in abundance, especially in the neighbourhood of the nerves.

It is a pretty general character of these elements that they take the stain of anilin colours and retain it on treatment with alcohol with greater energy than other tissue cells. And further (at least so far as regards the true Mastzellen of Ehrlich) that in successful preparations they show the nucleus unstained, the general mass of cytoplasm unstained or but slightly coloured, and, in the cytoplasm, the characteristic granules very intensely stained.

The staining reactions of the granules are very similar to those of bacteria. In order to distinguish them from these it may be observed that the stain of anilins is removed from the granules, but not from bacteria, by treatment with a weak solution of carbonate of potassium, (SQUIRE, *Methods and Formulæ*, p. 46). EHRLICH distinguishes no less than five kinds of granulations, recognisable by certain differences in their staining reactions. It will be remembered that according to a generally accepted principle of classification, due to Ehrlich (*Arch. f. Anat. u. Phys.*, Phys. Abth., 1879, pp. 167 and 572), anilin dyes are divided into two chief classes, viz. the "basic" dyes, or those in which the colouring matter plays the part of a base combined with a colourless acid; and the "acid" dyes, in which the colouring matter plays the part of an acid

in the compound; to which may be added a small group of "neutral" dyes. The basic dyes are in general nuclear stains,—that is, they exhibit a marked affinity for chromatin, as methyl green, for instance; and are also in general specific bacteria stains. The acid dyes have opposite affinities and are in general plasma stains, as eosin, for instance. The cell-granules in question have been classified by Ehrlich, according to their respective degrees of affinity for one or the other of these two groups of dyes, into (1) α granulations, or eosinophilous granulations, being such as in a mixture of dyes select the most acid stain there present; (2) the β granulations, or amphophilous granules, being such as in a mixture of basic and acid dyes take up both; (3) the γ or basophilous granules, being such as take up basic dyes only (such are the granules of Mastzellen; (4) the δ granulations, also basophilous; and (5) the ε granulations, or neutrophilous granules, being such as take up neutral anilin dyes, such as methyl blue and "acid" fuchsin (acid fuchsin is a weakly acid dye). I do not propose to enter minutely into the subject of these reactions, as it is one that cannot profitably be treated apart from the histology of the elements in question, and will confine myself to mentioning a few well-known methods.

789. EHRLICH'S "**Mastzellen**" (*Arch. f. mik. Anat.*, 1876, p. 263).—*First Method.*—Stain with neutral dahlia, and wash out with acidified water, and mount as directed *supra*, § 103. If there be any Mastzellen in the preparation they will be brought out by the superior energy with which they take the stain.

Second Method.—It may be desirable to obtain a *specific* stain of the plasma-cells alone, which may be done as follows:

The tissues must first be well hardened in strong alcohol (chromic acid and its salts must be avoided). They are then placed for at least twelve hours in a staining fluid composed of—

Absolute alcohol	50 c.c.
Aqua	100 c.c.
Acid. acet. glacial	12½ c.c.

—to which has been added enough dahlia to give an almost saturated solution. After staining, the preparations are

transferred to alcohol, which washes out the stain from all but the plasma-cells, and may then be mounted in the resin-turpentine solution.

Mucus-cells and fat-cells are also sometimes stained by these solutions.

Other Media.—In a similar way other soluble anilins may be employed (in the form of a fluid containing 7½ per cent. of acetic acid),—primula, iodine violet, methyl violet, purpurin, safranin, fuchsin; of these, methyl violet gives the best results.

790. Plasma-cells (Nordmann, *Beitr. z. Kenntniss d. Mastzellen, Inauguraldiss.*, Helmstedt, 1884).—Nordmann finds it useful to employ a solution of vesuvin containing 4 to 5 per cent. of hydrochloric acid. Sections should remain for a few minutes in the solution, and then be dehydrated with absolute alcohol. The paper quoted contains a detailed discussion of the microchemical reactions of granule-cells.

791. "Mastzellen."—Schiefferdecker (Schiefferdecker and Kossel's *Gewebelehre*, p. 329) recommends the following, after Orth. A piece of mesentery of a rat is brought into a solution of gentian violet in anilin water (§ 101), which is carefully heated over a flame until vapour begins to be given off, and then allowed to remain for a couple of hours (or the heating may be omitted, and the preparation allowed to stand for twenty-four hours). It is then rinsed in water, washed out until almost colourless in hydrochloric acid alcohol, rinsed in water, counterstained (if desired) in carmine, and mounted in balsam. Nuclei red, granules blue.

792. Plasma Cells and Mastzellen.—Unna, in his paper, *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 475, gives the following:

A. *For Plasma Cells.*

Methylen blue	1·0
Caustic potash	0·05
Distilled water	100·0

Add a few drops of this to ten, fifty, or one hundred vols. of anilin water (§ 101) in a watch-glass, and stain (alcohol material, or at most sublimate and alcohol material, not chromic material) for half an hour, several hours, or over-

night. Dehydrate rapidly in absolute alcohol, differentiate in creosol (details not given), rinse in xylol, and mount in balsam.

B. *General Stain, also bringing out Plasma Cells.*

Methylen blue	1·0
Carbonate of potash	1·0
Distilled water	100·0
Alcohol	20·0

Heat on a water-bath until reduced to 100·0. Use for staining undiluted, or diluted with one vol. of anilin water. Differentiate (details not given) with glycol, styron, or creosol. Mastzellen are not differentiated.

C. *Stain giving Red Mastzellen with Blue Plasma Cells.*

Methylen blue	1·0
Kali Carbon. (natron carbon, ammon. carbon)	1·0
Aq. dest. (Aq. carbolisata, chloroforma)	100·0

Dilute about 100-fold, stain slowly, treat with 70 to 80 per cent. alcohol, differentiate in styron, and bring through bergamot oil or xylol into balsam. In this process the granules of the Mastzellen stain red in consequence of the formation of *methylen red* in the staining bath.

In a paper "Ueber Plasmazellen, insbesondere bei Lupus" (for which see *Monatschr. f. prakt. Dermatol.*, xii, 1891, p. 296, or *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 92), UNNA gives some directions concerning the process of differentiation with creosol. Creosol only differentiates the stain, it does not dehydrate the sections. Sections should, therefore, first be dried with blotting-paper, and should then be treated with absolute alcohol for a few seconds, or with anilin oil before applying the creosol. The time required for differentiation in the creosol is from a few minutes to a few hours. When the proper stage of differentiation is attained, it should be fixed with xylol and the sections mounted. The method shows Mastzellen of a cherry-red tone.

In a paper, l. c., xiii, 1891, p. 364 (see *Zeit. f. wiss. Mik.*, xi, 1, 1892, p. 89), VAN DER SPEK and UNNA describe some further experiments. Maintaining the method of staining given above under A, they find that besides the differentiating agents given under B, a lengthy series of other reagents

are available. In all of them Mastzellen are distinguishable from plasma cells by the reddish coloration of their granules.

793. Plasma Cells and Mastzellen.—Bergonzini (*Anat. Anz.*, vi, 1891, pp. 595—600; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 95) gives the following:—Mix 1 volume of 0·2 per cent. solution of Säurefuchsin with 2 volumes of a like solution of methyl green, and 2 volumes of a like solution of gold-orange, and filter through cotton wool. Stain alcohol or sublimate sections (after washing in water) for three to four minutes, wash for one or two minutes in water, bring into absolute alcohol for two minutes, clear in bergamot oil or pure creosote, wash in turpentine, and mount in balsam.

One sort of gold-orange precipitates methyl green, and therefore cannot be used in this mixture. Orange G may be used instead (thus giving something very like Ehrlich-Biondi mixture), but in this case the acidophilous granules will stain greyish instead of red or orange. Basophilous granules ought to be green, weakly acidophilous granules red, strongly acidophilous ones orange or brown, nuclei always green. Other details l. c.

794. Plasma Cells.—Jadassohn (*Arch. f. Dermatol. u. Syphilis*, Ergänzungsheft 1, 1892, p. 58; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 226) recommends staining for nottoo long in a 1 : 2000 strongly alkaline or borax solution of Thionin, and washing out with acidulated water. Unfortunately this colour is, I believe, no longer obtainable in commerce.

795. Elastic Tissue.—Two of the most salient characters of elastic fibres are that they have a great affinity for osmium, staining with much more rapidity than most other tissue-elements, and that they are not changed by caustic soda or potash. A further character is that they have a great affinity for certain anilin dyes, especially Victoria blue.

For a review of the older methods of Balzer, Unna, Lustgarten, and Herxheimer, see the paper by Martinotti in *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 31.

Lustgarten's process, which is simple, and gives very good results, has been given in § 104. The colour used by him was called "Victoriablau 4 B," and this is probably an important detail.

But perhaps the best method is that of Martinotti (l. c.). Fix in a chromic liquid, wash, stain for forty-eight hours in strong (5 per cent. Pfitzner's) solution of safranin, wash, dehydrate, clear, and mount in balsam. Elastic fibres are

stained of an intense black, the rest of the preparation showing the usual characters of a safranin stain.

The staining will be performed quicker if it be done at the temperature of an incubating stove (GRIESBACH, *ibid.*, iv, 1887, p. 442). And FERRIA (*ibid.*, v, 3, 1888, p. 342) says that clearer preparations will be obtained if the sections be left for a long time, say twenty-four hours, in the alcohol, or be treated for a short time with very dilute alcoholic solution of caustic potash. This decolourises more completely the ground of the preparations.

Another safranin method, which seems to have the fault of requiring a too minute attention to details, is that of MIBELLI, see *Mon. zool. italiano*, 1, p. 17, or *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 225 (the report in *Journ. Roy. Mic. Soc.*, 1890, p. 803, is vitiated by a misprint).

UNNA'S **Orcein Method** (*Monatschr. f. prakt. Dermatol.*, xii, 1891, p. 394; *Zeit. f. wiss. Mik.*, ix, 1, p. 94) is as follows :— Dissolve 0·1 grm. of Grübler's orcein in 20 grms. of 95 per cent. alcohol and 5 grms. of water, and 0·1 grm. of concentrated hydrochloric acid in a like mixture of alcohol and water. Take six to ten watch-glasses, and pour into each 10 drops of the stain. Acidify them with the acid mixture, beginning with 5 or 10 drops for the first glass and increasing in the proportion of 1 drop for each glass in succession. Put one or two sections into each watch-glass and stain for twelve hours. Examine in a drop of glycerin and choose the successful sections; they should show the elastic fibres of a saturated shiny brown on a lighter ground. Counter-stain nuclei if desired.

See also ZENTHOEFER, in Unna's *Dermatol. Studien*, 1892, or *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 509.

KÖPPEN (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 473; and vi, 1, 1890, p. 22) gives the following:—Stain sections of alcohol material for fifteen to twenty-four hours in a freshly prepared mixture of five parts concentrated solution of crystal-violet ("Krystallviolett"), and 105 parts of 5 per cent. aqueous solution of carbolic acid. Treat for two minutes with the usual solution of iodine in iodide of potassium, then for five minutes with 10 per cent. salt solution, and for fifteen seconds with 1 per cent. hydrochloric acid. Wash out in absolute alcohol, clear with terebene followed by xylol, and mount in balsam. Elastic fibres dark violet, stratum corneum of cutis and sheath of Henle of hairs being also stained.

BURCI (see *Journ. Roy. Mic. Soc.*, 1891, p. 831; and 1892, p. 292) stains sections for a minute or two in saturated alco-

holic solution of Aurantia, dehydrates in absolute alcohol, clears in clove oil, and mounts in balsam.

Wolters (see *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 360) mordants sections for twenty-four hours in a mixture of two parts 10 per cent. chloride of vanadium, and eight parts 8 per cent. acetate of aluminium, stains for twenty-four hours in an incubator in Kultschizky's hæmatoxylin, and differentiates in Weigert's ferricyanide mixture, or, after a short treatment with solution of chloride of iron, in water.

For a somewhat complicated method of Martinotti's, with nitrate of silver, which is, I think, of more theoretical than practical interest, see *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 521.

For the methods of Unna and Taenzer with nitrate of rosanilin salts, see *Monatsch. f. prakt. Dermat.*, vi, 1887; or Behrens, Kossel, u. Schieferdecker, *Das Mikroskop*, 1, p. 204.

796. Bone, Non-decalcified (Ranvier, *Traité*, p. 297).—Ranvier points out certain precautions that it is necessary to take in the preparation of sections of dry bone. In general, the bones furnished by "naturalists," or procured in anatomical theatres, contain spots of fatty substance that prevent good preparations from being made. Such spots are formed when bones are allowed to dry before being put into water for maceration; when a bone is left to dry the fat of the medullary canals infiltrates its substance as fast as its water evaporates.

Bones should be plunged into water as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water; spongy bones should be submitted to hydrotomy. This may be done as follows:—An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure.

As soon as the bones, whether compact or spongy, have been freed from their medullary substance they are put to macerate. The maceration should be continued for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones

may be left to dry. When dry, they should be of an ivory whiteness, and their surfaces exposed by cutting of a uniform dulness.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone. Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but see VON KOCH'S copal process, *ante*, § 311, and EHRENBAUM'S colophonium process, § 312).

WEIL (*Zeit. f. wiss. Mik.*, v. 2, 1888, p. 200) gives a process similar to the foregoing, rough sections or portions of bone and teeth being penetrated with chloroform balsam, which is hardened in an oven, or over a water-bath, and then ground.

RÖSE (*Anat. Anz.*, vii, 1892, pp. 512—519 ; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 506) points out some precautions that it is well to take. He penetrates first with a mixture of cedar oil and xylol, then with pure xylol, and imbeds in solution of dammar in chloroform or xylol. The method can be combined with Golgi's impregnation.

NEALEY (*Amer. Mon. Mic. Journ.*, 1884, p. 142 ; *Journ. Roy. Mic. Soc.*, 1885, p. 348) says that perfectly fresh portions of bone or teeth may be ground with emery on a dentist's lathe, and good sections, with the soft parts *in situ*, obtained in half an hour.

WHITE (*Journ. Roy. Mic. Soc.*, 1891, p. 307) recommends the following :—Sections of osseous or dental tissue should be cut or ground down moderately thin, and soaked in ether for twenty-four hours or more. They should then be put for two or three days into a thin solution of collodion stained with fuchsin (made by dissolving the dye in methylated spirit, adding the requisite quantity of ether, then the pyroxylin). The sections are then put into spirit to harden the collodion. After this they are ground down to the requisite thinness

between two plates of old ground glass, with water and pumice powder, and mounted, *surface dry*, in stiff balsam or styrax, care being taken to use as little heat as possible. Lacunæ, canaliculi, and dentinal tubuli are found infiltrated by the coloured collodion.

Matschinsky (*Arch. f. mik. Anat.*, xxxix, 1892, p. 151 ; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 353) prepares sections by soaking in gum and rubbing down as recommended by Ranvier. When sufficiently thin, the gum is removed by means of water, the sections dried, put for half an hour into benzin or ether, and stained in a saturated aqueous solution of some anilin dye, from one to seven days according to the age of the bone. They are then washed in water, further ground down, put for twenty-four hours into alcohol, and mounted by the aid of heat in thickened balsam. The result is a stain of the ground substance, of varying degrees of intensity.

Hopewell-Smith (*Journ. Brit. Dent. Ass.*, xi, 1890, p. 310 ; *Journ. Roy. Mic. Soc.*, 1890, p. 529) says that for preparing sections of teeth showing odontoblasts *in situ* the best plan is to take embryonic tissues. A lower jaw of an embryonic kitten or pup may be taken, and hardened in solution of Müller followed by alcohol, then cut with a freezing microtome. The knife cuts quite easily the thin cap of semi-calcified dentine and bone.

797. Bone and Teeth, Decalcification Methods.—Besides the processes given in Chap. XXIV, attention may be called to the following :

Hopewell-Smith (*Trans. Odont. Soc.*, xxiv, 1891, p. 20; *Journ. Roy. Mic. Soc.*, 1892, p. 433).—Harden a newly-extracted tooth in solution of Müller (three to four weeks) followed by alcohol. Wash in water, and seal up apical foramen with collodion. Decalcify as directed in § 565. Wash with distilled water. Divide with a razor into several pieces, wash them in water, and put them for twelve to fifteen hours into gum mucilage (B.P.). Cut with a freezing microtome, and stain with orange-rubin, gold-chloride, borax-carmine, or by Weigert's method. Dehydrate, clear with cedar oil, and mount in balsam.

798. Lepkowsky (*Anat. Anz.*, vii, 1892, p. 274 ; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 355) recommends the following as giving

a superior preservation of the structures. Sections of fresh teeth, not thicker than 0·75 mm., are made with a saw, and put into a mixture of six parts 1 per cent. gold chloride and three parts pure formic acid, for twenty-four hours. Wash with distilled water, and reduce for twenty-four hours in glycerin gum solution. Pass through water and alcohol and imbed in celloidin or paraffin, and cut. The dentinal canaliculi are shown stained.

Bone can be treated in the same way.

For UNDERWOOD'S gold process for teeth see § 228.

799. VIVANTE (*Intern. Monatssch. f. Anat. u. Phys.*, ix, 1892, p. 394; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 351) has made out that thin specimens of bone can be successfully treated by Golgi's bichromate of silver process. He places portions of frontal bone of four to six months calves, which are not more than 3 to 4 mm. thick, for eight days in solution of Müller, then in the osmium bichromate mixture, and then in the silver solution. After impregnation the specimens should be decalcified, which may be done by putting them for twenty days into von Ebner's mixture (§ 563), after which they should be well washed with water and brought into solution of carbonate of soda, and finally imbedded in paraffin.

Another method recommended by Vivante is as follows:—Fix for five or six days in solution of Flemming, wash and decalcify with von Ebner's mixture, wash, treat with carbonate of soda, imbed in paraffin, cut, and stain the sections for an hour in 0·2 per cent. solution of quinoleïn blue (§ 126). Wash out in equal parts of alcohol and water followed by pure water, dry the sections in a stove at 40° C. (both alcohol and glycerin must be avoided), clear with bergamot oil, and mount in damar. Nuclei violet, protoplasm and ground substance different shades of blue.

800. Bone, Decalcified (FLEMMING, *Zeit. f. wiss. Mik.*, 1886, p. 47).—Sections of delcacified bone are made with the free hand. They are soaked in water, and brought in a drop of water on to a glass plate, where they are spread out flat. The excess of water is removed with blotting-paper, and the sections are covered with another glass plate, to prevent them from rolling. The whole is brought into a plate and covered with alcohol. After the lapse of half an hour the sections have become fixed in the flat position, and may be brought into absolute alcohol without risk of their rolling. To mount them, wash them with fresh alcohol (which may be followed

by ether); lay them again flat on glass, and cover them with a double layer of blotting-paper and a somewhat heavy glass plate, and let them dry for a day in the air or in a stove. When they are dry, put a drop of melted balsam on a slide, and let it spread out flat and cool. Prepare a thin glass cover in the same way, put the section on the prepared slide, cover it with the prepared cover, put on a clip, and warm.

By this process sections can be very expeditiously prepared, which show the lacunar system injected with air in quite as instructive a manner as non-decalcified sections.

Kölliker (*Zeit. f. wiss. Zool.*, xliv, 1886, p. 662) recommends the following process for the demonstration of the fibres of Sharpey in decalcified bone. Sections are treated with concentrated acetic acid until they become transparent, and are then put for one quarter to one minute into a concentrated solution of indigo-carmine, then washed in water and mounted in glycerin or balsam. In successful preparations the fibres of Sharpey appear stained of a pale or dark *red*, the remaining bone-substance blue.

801. Cartilage (and Decalcified Bone).—For an excellent discussion (especially as regards staining) of the methods that have been recently recommended for these objects, see the exhaustive paper of Schaffer in *Zeit. f. wiss. Mik.*, v. 1, 1888, which gives in sufficient detail all the methods in question. The following appear to be the best :—

Ranvier's *purpurin method.* This has been given in detail in § 201.

Schaffer's *safranin method.* This method is due in its principle to Bouma (*Centralb. f. d. med. Wiss.*, 1883, p. 866). I give it in the form to which it has been brought by the careful study of Schaffer (*Zeit. f. wiss. Mik.*, v. 1, 1888, p. 17). Sections of bone decalcified with nitric acid (chromic acid may be used, but the stain will be less brilliantly contrasted) are stained for half an hour to one hour in 0·05 per cent. aqueous solution of safranin, washed with water, put for two or three hours in 0·1 per cent. solution of corrosive sublimate, and examined in glycerin. In order to make permanent preparations, the sections on removal from the sublimate are rinsed with alcohol, pressed on to a slide with filter-

paper, cleared *for a long time* in bergamot oil or clove oil, and mounted in xylol balsam.

This is a *double* stain; cartilage, orange; bone, uncoloured (or green in chromic objects); marrow, red.

BAYERL'S *method for ossifying cartilage* (*Arch. f. mik. Anat.*, 1885, p. 35) is as follows:—Portions of ossified cartilage are decalcified as directed § 564, cut in paraffin, and stained in Merkel's borax-carmine and indigo-carmine mixture, as directed § 242, and mounted in balsam.

MAX FLESCH (*Zeit. f. wiss. Mik.*, 1885, p. 351) particularly recommends this process for the study of the development of dental tissue.

MOERNER (*Skandinavisches Arch. f. Physiol.*, i, 1889, p. 216; *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 508) gives several stains for tracheal cartilage, chiefly as microchemical tests. 1. Stains for the trabeculæ ("Balkennetz").—*a*. Sections (of alcohol material, I gather) put for half an hour into concentrated aqueous solution of Tropæolin 000, No. 2 (Schuchardt's), and washed out in water until only the trabeculæ remain coloured. *b*. Stain for a few minutes in concentrated aqueous solution of Sulphindigotate of Potash (*not soda*), and wash out as above.

2. Stains for "Chondrinballen" (groups of cartilage-cells). *a*. Stain for half a minute to two minutes in 0·15 per cent. aqueous solution of methyl violet, rinse in water, and wash out in 10 per cent. acetic acid until the trabeculæ are decoloured. "Chondrinballen" blue on a colourless ground. *b*. Stain as above, using a 0·15 per cent. solution of anilin red, and wash out as before.

3. Combined methods. *a*. Stain as in 1 *a*, then as in 2 *a*, dehydrate quickly, and clear in clove oil. *b*. Stain as in 1 *b*, then as in 3 *a*, but using anilin red instead of methyl violet.

See a critique of these methods by WOLTERS in *Arch. f. mik. Anat.*, xxxvii, 1891, p. 492; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 383; also, on the whole subject of cartilage, see SCHIEFFERDECKER'S *Gewebelehre*, p. 331.

Blood.

802. It might be supposed that for the study of blood it would suffice to prick a finger, place a drop of blood on a slide, cover, and examine it. But this is by no means the

case. "*Blut ist ein ganz besonderer Saft,*"* and will not yield up its secrets to such simple wooing. The technique of blood is most elaborate; see, for instance, the voluminous work of HAYEM, *Du sang et de ses altérations anatomiques*, pp. 1035, with 126 figures, Paris, Masson, 1889 (a report of over twenty pages on this important work is contained in *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 330, *et seq.*); LÖWIT, *Sitzb. k. Acad. Wiss. Wien*, 3, lxxxviii, 1883; xcii, 1885; xcv, 1887; Ziegler's *Beitr. z. path. Anat.*, x, 1891, p. 214; *Zeit. f. wiss. Mik.*, vi, 1889, pp. 74, 76; viii, 3, 1891, p. 371; *Arch. f. mik. Anat.*, xxxviii, 1891, p. 524; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 233; *Studien zur Physiol. u. Path. d. Blutes u. d. Lymphe*, Jena, Fischer, 1892; EHRLICH, *Zeit. f. klin. Medicin*, i, 1880, 3, p. 558; *Zeit. f. wiss. Mik.*, i, 1884, p. 382, and other papers; MUELLER, *Sitzb. k. Acad. Wiss. Wien*, xcviii, 3, p. 219; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 365; GRIESBACH, *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 326; and many other investigators.

It is out of the question for me to attempt to abstract these memoirs in the space at my disposal, so I must confine myself to giving a few methods that may be useful to the general student, referring the specialist to the original papers.

803. Fixing and Preserving Methods.—The time-honoured process of drying drops of blood over a flame gives rise to great deformation of the elements, and should be abandoned as far as possible. It is better to mix the blood at once with some fixing and preserving medium, and study it as a fluid mount.

Most recent authors (BIONDI, MOSSO, MAX FLESCH) are agreed that by far the most faithful fixing agent for blood-corpuscles is osmic acid. A drop or two of blood (Biondi recommends two drops exactly) is mixed with 5 c.c. of osmic acid solution, and allowed to remain in it for from one to twenty-four hours. The exact degree of concentration of the osmium solution is a somewhat important point, and must be made out by experiment for each form. As a rule it should be strong, 1 to 2 per cent. According to Biondi, 2 per cent. is best. Fixed specimens may be preserved for use in acetate of potash solution (MAX FLESCH, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 83).

* Goethe's *Faust*, i, 4, line 1387.

Griesbach also (op. cit., p. 328) prefers osmic acid, not only as being a first-rate fixing agent, but because it can be combined with certain stains without decomposing them. He mentions methyl green, methyl violet, crystal violet, safranin, eosin, Säurefuchsin, rhodamin, and iodine in potassic iodide.

Rossi (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 475) advises a mixture of equal parts of 1 per cent. osmic acid, water, and strong solution of methyl green, permanent mounts being made by means of glycerin cautiously added.

The mercurial liquids of Pacini (§ 380), especially Nos. 2 and 3, are good. Hayem (op. cit.; see also *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 335) has a similar formula, viz. sublimate 0·5, salt 1, sulphate of soda 5, and water 200. This should be mixed with blood in the proportion of about 1:100. Eosin may be added to it. Löwit's formula (*Sitzb. k. Acad. Wiss. Wien*, xcv, 3, p. 129; *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 75) consists of 5 c.c. cold saturated sublimate solution, 5 grms. sulphate of soda, 2 grms. salt, and 300 c.c. water. Mosso finds, however, that both of these are too weak in sublimate.

Of course other well-tried fixing fluids, such as Flemming's solution, or Hermann's, may also be used for blood.

804. Stains for Blood.—Blood prepared as above can be satisfactorily stained with many of the usual reagents.

Eosin stains rose-red all parts of blood-corpuscles that contain hæmoglobin (see Wissowsky, *Arch. f. mik. Anat.*, 1876, p. 479); parts that do not contain hæmoglobin, such as the nucleus, remaining unstained. This suggests double-staining with eosin and hæmatoxylin.

Wissowsky (l. c.) stains in a solution of equal parts of eosin and alum in 200 parts of alcohol, and then with hæmatoxylin.

Moore (*The Microscope*, 1882, p. 73; *Journ. Roy. Mic. Soc.*, 1882, p. 714) stains for three minutes in a similar solution without the alum, washes, and stains for two minutes in a 1 per cent. aqueous solution of methyl green. Red corpuscles, red; nuclei and white corpuscles, bluish green.

Merkel's *carmine and indigo-carmine* stain has been discussed above (§ 242).

Fresh (unfixed) blood is perhaps best treated as follows (Bizzozero and Torre, *Archivio per le Scienze mediche*, vol. iv, No. 18, 1880, p. 390) :—Dilute a drop of blood with 0·75 per cent. salt solution in which has been dissolved a little *methyl violet*. This liquid in no wise affects the form of the elements, stains intensely the nucleus of the red corpuscles, and, in the white, stains the nucleus intensely, and the protoplasm less intensely. May be used for the study of bone-marrow and spleen.

For the staining of the blood-plates of Bizzozero, this observer (*Arch. f. path. Anat. u. Phys.; Zeit. f. wiss. Mik.*, 1884, p. 389) employs a 0·02 per cent. solution of methyl violet in salt solution, or a 1 : 3000 solution of gentian violet.

Toison (*Journ. Sci. med. de Lille*, fév., 1885; *Zeit. f. wiss. Mik.*, 1885, p. 398) recommends that blood be mixed with the following fluid:

Distilled water . . .	160 c.c.
Glycerin (neutral, 30° Baumé) .	30 c.c.
Pure sulphate of sodium . .	8 grammes.
Pure chloride of sodium . .	1 gramme.
Methyl violet 5 B . . .	0·25 „

(The methyl violet is to be dissolved in the glycerin with one half of the water added to it; the two salts are to be dissolved in the other half of the water, and the two solutions are to be mixed and filtered.) White blood-corpuscles stain in this medium in five or ten minutes; the maximum of coloration is attained in from twenty to thirty minutes. White blood-corpuscles, violet; red blood-corpuscles, greenish.

Ferrier's liquid is said to have a sp. gr. similar to that of liquor sanguinis. Fuchsin, 1 grm.; water, 150 c.c.; rectified spirit, 50 c.c.; dissolve, and add glycerin, 200 c.c. (from Squire's *Methods and Formulæ*, p. 39).

Leclerq's fuchsin followed by malachite green, or Congo followed by gentian and eosin, see *Bull. Soc. Belge de Mic.*, xvi, 1890, p. 61; or *Journ. Roy. Mic. Soc.*, 1890, p. 675.

Dekhuysen's methylen blue and acid fuchsin mixture, see *Verhandl. Anat. Gesellsch.*, 1892, p. 90; or *Journ. Roy. Mic. Soc.*, 1893, p. 116.

Griesbach, besides the stains mentioned in the last § as being capable of being combined with an osmic acid fixing solution,

recommends a double stain by means of a mixture of solutions of methyl green and rhodamin added to osmic acid solution (1 per cent., the dyes in concentrated solution, but the proportions of the mixture not given).

It goes without saying that the EHRLICH-BIONDI mixture (§§ 259 and 639) will be found a most valuable reagent in many hæmatological researches. LÖWIT (Ziegler's *Bietr. z. path. Anat.*, &c., x, 1891, p. 214; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 371) obtained instructive results by staining sublimate preparations for one to two minutes in a *concentrated* solution, and examining in water or glycerin.

For details as to the reactions of the granules of leucocytes and of Löwit's "pyrenogenous" corpuscles, see the original paper; also EHRLICH'S "Methodologische Beitr. z. Physiol., &c., der Leucocyten," in *Zeit. f. klin. Med.*, i, 1880, 3, p. 558; cf. *Zeit. f. wiss. Mik.*, i, 1884, p. 382, and later papers of Löwit in *Anat. Anz.*, vi, 1891, p. 344, and *Arch. f. mik. Anat.*, xxxviii, 1891, p. 524 (*Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 233).

FOÀ (*Festschr. R. Virchow gewidm.*, &c., 1891, i, p. 481; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 227) proceeds as follows:— Preparations (either coagulated blood or small pieces of hæmatopoietic organs) are fixed in a solution of 2 grms. sublimate in 100 grms. of liquid of Müller (the sublimate fixes the figured structures of protoplasm and nuclei, the Müller fixes the hæmoglobin). They are imbedded in paraffin, and sections made and stained for one to three minutes in the following mixture:

Aq. dest., about	100 grms.
Böhmer's hæmatoxylin	25 „
1 per cent. alcoholic aqueous safranin solution	20 „

Wash out in water, followed by weak alcoholic solution of picric acid, dehydrate, and mount in balsam. Several other methods are also mentioned in the same paper.

The elaborate paper of MUELLER mentioned in § 802 is too rich in detail to bear abstracting here. A novelty in it is the impregnation of cover-glass preparations with gold chloride by Ranvier's formic acid process.

805. Demonstration of Blood-plates of Bizzozero (KEMP, *Studies fr. the Biol. Lab. Johns Hopkins Univ.*, May, 1886, iii, No. 6; *Nature*, 1886, p. 132).—The mere demonstration of the blood-plates of Bizzozero is easy enough. A somewhat large drop of blood is placed on a slide, and quickly washed with a small stream of normal salt solution. The blood-plates are not

washed away, because they have the property of adhering to glass; and on bringing the slide under the microscope they will be seen in large numbers. If it be desired to make permanent preparations of them, they should first be fixed. This is done by putting a drop of osmic acid solution on the finger before pricking it.

For BIZZOZERO'S recent methods for the *numeration* of these elements and for the study of their regeneration, see his paper in *Festschr. R. Virchow gewidm.*, &c., 1, 1891, p. 459; or the report of the methods in *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 229.

For the application of some *digestion methods* to the study of blood-plates, see LILIENFELD, *Arch. f. Anat. u. Physiol.*, Physiol. Abth., 1892, p. 115; or *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 363.

806. BIONDI'S **Section Method for Blood** (*Arch. f. mik. Anat.*, xxxi, 1888, p. 103).—None of the foregoing methods are perfectly satisfactory as regards the preservation of the elements of blood without deformation, and at the same time in a perfectly permanent manner. Biondi's ingenious process does this.

Blood is fixed with osmic acid solution as described above, § 803. Four or five drops of the mixture of blood and osmium solution are then mixed with agar-agar jelly melted at 35° to 37° C. The whole is allowed to cool, and the mass is put to harden in alcohol of 85 per cent. After a few days the mass will have attained a consistence that allows of its being imbedded in pith and cut with a microtome. The sections are treated according to the usual methods. The best stains are obtained with methyl green, methylen blue, fuchsin, and safranin. Methyl green and eosin is also a good combination. After staining, the sections are cleared and mounted in balsam in the usual way.

Thinner sections can be obtained if the agar-agar mass be imbedded in paraffin in the usual way, instead of in pith.

Instructions, too long to be abstracted here, are given for the preparation of a suitable agar-agar mass. It may be obtained ready prepared from Herrn König, 29, Dorotheenstrasse, Berlin. Celloidin, and others of the usual imbedding masses, were tried, but without success. SCHIEFFERDECKER, however, says that celloidin may be employed (see his *Gewebelehre*, p. 389).

For further details the English reader may consult the *Journ. Roy. Mic. Soc.*, 1888, pp. 313, 659.

This is undoubtedly a valuable method, and is capable of extension to the study of other animal fluids besides blood.

Glands.

807. Mucin.—It has already been stated that the blue solutions of hæmatoxylin have a special affinity for mucin. For the demonstration of mucus gland-cells the following process is, therefore, recommended by FLEMMING (*Zeit. f.*

wiss. Mik., 1885, p. 518) :—Stain sections first with hæmatoxylin of Heidenhain, and afterwards with hæmatoxylin of Delafield or Böhmer. The mucus cells are shown stained violet.

Hoyer, who has made a special study of the staining reactions of mucin in tissues (*Arch. f. mik. Anat.*, xxxvi, 1890, p. 310; see also *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 67), has the following conclusions :

The mucin of mucus cells and goblet cells, both of Vertebrates and Invertebrates, stains with basic tar colours, but not with acid tar colours (see above, § 788). More or less specific stains of mucin are obtained, for instance, with hydrochlorate or nitrate of rosanilin, commercial fuchsin, Grübler's "neutral" fuchsin ("*n. Unna*"), magenta, Magdala red, iodine green, methyl green, dahlia, methyl violet, gentian, iodine violet, crystal violet, Victoria blue. A similar reaction is obtained with alum-hæmatoxylin solutions, whilst carmine behaves like the acid coal-tar dyes, and affords no stain.

Hoyer obtained his best results by means of thionin (violet of Lauth), which gives a double stain, the tissues blue, the mucin elements ruddy violet. This dye is unfortunately no longer to be found in commerce ; but the dye called amethyst, prepared by Geigy and Co., of Bâle, is a good succedaneum, and so are toluidin blue (obtainable from Grübler) or the phenylen blue of Oehler in Offenbach, and the p-phenylen blue of the Höchst manufactory. Like thionin, all these give metachromatic stains.

Results less brilliant than those given by the above-mentioned stains, but nevertheless excellent, are obtained by means of methylen blue or Bismarck brown. Methylen green and safranin also give good reactions, but are somewhat inconstant in their effects. Methylen blue is particularly useful from its power of bringing out the merest traces of mucin.

All of these colours may be used in the same way. Specimens should be fixed for two to eight hours in 5 per cent. sublimate solution, imbedded in paraffin, cut, and the sections stained for five to fifteen minutes in a very dilute aqueous solution of the dye (two drops of saturated solution to 5 c.c. of water).

It is theoretically interesting to observe that hyaline cartilage, the jelly of Wharton, and the Mastzellen of Ehrlich give the same reactions with basic dyes as mucin does, even their metachromatic reactions being identical.

These conclusions had already been in part formulated by SUSSDORF (*Deutsche Zeit. f. Thiermed.*, xiv, pp. 345, 359; see *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 205).

See also the important series of papers by BIZZOZERO, "*Sulle ghiandole tubulari del tubo gastro-enterico*," &c., in the *Atti R. Accad. di Sci. di Torino*, 1889 to 1892; reports in *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 61; and ix, 2, 1892, p. 219. As regards the safranin reaction, it is well to note that it is not obtained with all brands of the dye; that of Bindschedler and Busch, in Bâle, gives it, whilst safranin 0 of Grübler does not. For the distinctive reactions of old and young mucin see the original, or the last-quoted report of the *Zeit. f. wiss. Mik.*

808. "Reticulum" of Mucus Cells.—This was first demonstrated by SCHIEFFERDECKER (*Arch. f. mik. Anat.*, 1884, Heft 3) by means of anilin green. This reaction has given rise to a long polemic between SCHIEFFERDECKER and LIST, for which see *Zeit. f. wiss. Mik.*, 1885, pp. 51, 222, 223. According to List, the reticula in question stain in an equally specific way in methyl green, in Bismarck brown, in nitrate of rosanilin (of 0·0001 per cent., ten to fifteen minutes; see op. cit., iii, 3, 1886, p. 393), and in List's double-stains (§§ 262, 263). Schiefferdecker, on the contrary, maintains that the reticula demonstrated by the methods of List are not identical with those demonstrated by anilin green.

809. Goblet Cells.—So far as these contain mucin they give the reactions above described (see FLEMMING, *Zeit. f. wiss. Mik.*, 1885, p. 519; and PAULSEN, *ibid.*, p. 520). But the reactions appear to be different for different animals. Thus PANETH (*Arch. f. mik. Anat.*, xxxi, 1888, p. 113, *et seq.*) found that in the small intestine of the mouse the contents of the goblet cells did not stain with Böhmer's hæmatoxylin. And the goblet cells of the small intestine of man did not stain with safranin.

RANVIER, in a paper too long to be abstracted here (*Comptes rend.*, 1887, 3, p. 145; see also *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 233), describes a specific reaction of perruthenic acid (RuO_4) on goblet cells. By treating the pharyngeal mucosa of the frog first for ten to twelve hours with vapour of osmium, and then for three minutes with vapours of perruthenic acid, the goblet cells are brought out with remarkable distinctness. The contained

mucigen is stained black, but the vacuoles are unstained. Since perruthenic acid is very rapidly reduced by organic matter, Ranvier regards this reaction as a proof that the vacuoles do not contain any organic substance, but probably only water and inorganic salts.

For detailed instructions for the study of goblet cells, see LIST, in *Arch. f. mik. Anat.*, xxvii, 1886, p. 481.

See also *ante*, §§ 246, 247, 252, 255.

810. Liver.—See hereon the important papers of RANVIER, "*Les membranes muqueuses et le syst. glandulaire*," in the *Journ. de Microgr.*, ix, x, 1885–6; IGACUSCHI, in *Arch. f. path. Anat.*, xcvii, p. 142, or *Zeit. f. wiss. Mik.*, 1885, p. 243 (gold process for study of fibrous networks); KUPFFER, *Sitzb. Ges. f. Morph.*, &c., München, Juli, 1889, or *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 506 (hæmatoxylin stain for demonstration of ultimate bile-ducts, and application of Golgi's silver bichromate method to the same object and to the study of fibrous networks); OPPEL, *Anat. Anz.*, v, 1890, p. 143; vi, 1891, p. 165; and *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 222; viii, 2, 1891, p. 224 (also concerning the application of Golgi's process to the above objects).

811. Other Methods for Glandular Structures.—Amongst numerous important papers that cannot be quoted here, see RANVIER, "*Le mécanisme de la sécrétion*," in *Journ. de Microgr.*, x, 1886–7, and the valuable papers of HEIDENHAIN in Pflüger's *Archiv*.

The peculiar applicability of the Ehrlich-Biondi stain, § 259, to this kind of work hardly needs pointing out.

Indigo, used as in § 241 or 242, may also be found very useful.

For salivary glands see § 252.

CHAPTER XXXII.

SOME ZOOLOGICAL METHODS.

Tunicata.

811. Fixation of Tunicata.—A method of SALVATORE LO BIANCO for killing simple Ascidians in an extended state has been given above, § 18. In the paper quoted below* this plan is recommended for *Ciona, Ascidia,* and *Rhopalea.* But many other forms, such as *Clavellina, Perophora, Phallusia, Molgula, Cynthia,* &c., should first be narcotised by treatment for from three to twelve hours with chloral hydrate† (1 : 1000 in sea water), then killed in chromo-acetic acid (chromic acid of 1 per cent. 100 parts, acetic acid 10 parts).

The compound Ascidians with contractile zooids are difficult to manage if one does not go the right way to work. The best process known to me is the following (due to VAN BENEDEN, kindly communicated to me by Dr. C. Maurice). Place the corms in clean sea water, and leave them alone for a few hours, in order that the zooids may become fully extended. Seize the corms with your fingers, and plunge them suddenly into glacial acetic acid. Leave them there for two, four, or six minutes, according to the size of the corms (which of

* A valuable paper giving an account of a number of the processes employed in the Naples Zoological Station for the preservation of marine animals has been published by SALVATORE LO BIANCO in *Mitth. zool. Stat. Neapel,* ix, 1890, p. 435. References to the work of S. LO BIANCO in the remainder of this chapter are to that paper. An abstract of it is contained in *Amer. Natural.*, xxiv, 1890, p. 856, and *Journ. Roy. Mic. Soc.*, 1891, p. 133, and a very full account in *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 54.

† A new narcotising process recently incroduced by my friend Dr. BROCHER, of Geneva, has been warmly recommended to me as being more certain in its action than those hitherto employed. It consists in treatment with monobromated camphor, which, in the case of small organisms at all events, may be exhibited by simply placing a few crystals in the water in which they are contained.

course you will have taken care to select of as small a size as possible). Take them out of the acid with your fingers (or in some manner that may dispense with the employment of steel instruments, which would blacken the tissues) and bring them into 50 per cent. alcohol. Wash them thoroughly in that, and then bring them in the usual way through successively stronger alcohols.

I most strongly recommend this process, which gives admirably preserved preparations quite free from any opacity either in the tissues or the tunic. The acid will not hurt the fingers if they be washed immediately.

S. Lo Bianco recommends for this group the chloral hydrate process, followed by fixation with sublimate or chromo-acetic acid.

Small pelagic Tunicates are very easily fixed with osmic acid or acid sublimate solution, with the exception of *Anchinia*. The not very numerous preparations I have made of this exceedingly delicate form have all been unsatisfactory. And some other similar forms may be found difficult. I have had a striking failure with *Salpa virgula*, which I fixed with "Flemming," and got a very poor preparation. The very similar *S. pinnata* is fixed perfectly in this medium.

Molluscoida.

812. Bryozoa.—For some methods of killing and fixing see §§ 7, 14, and 15. S. Lo Bianco employs for *Pedicellina* and *Loxosoma* the chloral hydrate method, fixing with sublimate. For *Flustra, Cellepora, Bugula, Zoobothrium*, he employs the alcohol method of Eisig, § 12.

Brocher's monobromated camphor, last section, may be useful for this group.

Mollusca.

813. Fixation of Mollusca.—Two groups at least amongst the Mollusca offer considerable difficulties in the way of fixation—Lamellibranchiata and Gastropoda.

If it be attempted to take living and normal Lamellibranchiata from the water they are contained in, in order to throw them into a fixing solution, they invariably withdraw their siphon and foot, shut their valves, and die in a state of contraction. And if it be attempted to open the shell by force

after death, the mantle is generally injured, and it is impossible to get the foot and siphon into the extended state. De Castellarnau (*La Estacion Zoolog. de Napoles*, Madrid, 1885) advises that they be killed by the method of Eisig and Andres described for Actiniæ in § 12. Before dying, the animals protrude largely their feet, siphons, branchiæ, and tentacles, and die with their shells open. They may be fixed as soon as insensibility has supervened, by bringing them into picro-sulphuric acid, or some other rapidly killing fixing agent.

In order to demonstrate the absence of the supposed aquiferous pores in the root of Lamellibranchiata, Fleischmann (*Zeit. f. wiss. Zool.*, xlii, 1885, p. 376) proceeds as follows :—A mussel is quickly seized at a moment when the foot is fully extended, and the two valves of the shell are forcibly pressed together, so as to prevent any flowing back towards the interior of the liquid contained in the foot. The foot may then be fixed by holding it for a few minutes in hot sublimate solution.

The same methods recommended for Lamellibranchiata sometimes give good results with Gastropoda. The asphyxiation method has been described in § 19.

S. Lo Bianco advises that Lamellibranchiata, Prosobranchiata, and, amongst the Heteropoda, Atlantidæ, be narcotised with 70 per cent. alcohol, § 12. Opisthobranchiata ought not to give much trouble, and I recommend sudden killing with liquid of Perényi, or the acetic acid method, § 811.*

For Pteropoda in general, liquid of Perényi. *Creseis* is a difficult form. S. Lo Bianco advises the alcohol method, § 12.

Note the hydroxylamin method of Hofer, § 16; also Brocher's monobromated camphor, § 811.

814. Terrestrial Gastropods.—The asphyxiation method has been described in § 19.

The quantity of mucus that exists in the integument of Gastropoda is often a serious obstacle in the way of preparation. Marchi (*Arch. f. mik. Anat.*, 1867, p. 204) finds that if a living Limax be thrown into moderately concentrated salt solution it will throw off enormous quantities of mucus, and die in a few hours. The epidermis will be found well preserved. If

* *Aplysia* may first be narcotised by subcutaneous injection of about 1 c.c. of a 5 to 10 per cent. solution of hydrochlorate of cocaïn (Robert, *Bull. Scient. de la France*, &c., 1890, p. 449; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 216).

the animal be thrown into osmic acid or Müller's solution, if I understand the writer justly, no secretion of mucus will occur.

815. Eyes of Gastropoda (Flemming, *Arch. f. mik. Anat.*, 1870, p. 441).—The first difficulty here is to obtain the excision of an exserted eye. It is impossible to sever the exserted peduncle in a living animal without its retracting at least partially before the cut is completed. Never mind that; make a rapid cut at the base, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmium, in alcohol, or in bichromate.

Carrière (*Zool. Anz.*, 1886, p. 221) gives the following instructions:—Remove the eye, together with a portion of the tentacle, and fix it by exposing it for some minutes to vapour of osmium. Make sections according to the usual methods, and fix them on a slide with Schällibaum's collodion. Stain them with picro-carmine; or first depigment them by very careful treatment with *very dilute* eau de Javelle, and then stain with picro-carmine. Mount in dammar. Successful preparations show the tissues perfectly preserved; but Carrière has only been able to make the depigmentation process succeed with *Helix pomatia*; with Prosobranchiata he failed.

816. Eyes of Cephalopoda and Heteropoda (Grenacher, *Abh. naturf. Ges. Halle-a.-S.* Bd. xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244).—Fix in picro-sulphuric acid, or in a saturated solution of corrosive sublimate in picro-sulphuric acid (this mixture is especially useful for *Octopus*, *Eledone*, and *Sepia*, but does not succeed with the pelagic forms, such as *Loligo*, *Ommatostrephes*, and *Rossia*). Depigment the specimens with hydrochloric acid (in preference to the nitric acid used by Grenacher in former researches). The mixture § 583 may also be used. The operation of depigmentation may be combined with that of staining; if you stain with borax-carmine and wash out in the last-mentioned mixture the pigment will be found to be removed quicker than the stain is washed out. But this process is delicate, and requires a practised hand. The operation of depigmentation may be carried out on sections, but it is better to use portions of retina of 2 to 5 mm. in thickness. Grenacher mounted his preparations in castor oil, see § 425.

Similar methods are recommended by the same author for the eyes of **Heteropoda** (see *Abh. naturf. Ges. Halle-a.-S.*, 1886 ; *Zeit. f. wiss. Mik.*, 1886, p. 243).

817. Eyes of Chitonidæ (Moseley, *Quart. Journ. Mic. Sci.*, 1885, p. 40).—Moseley worked by decalcifying the shell and making sections. He places fragments of shell (of which the tissues have previously been hardened in strong alcohol) in 100 to 200 c.c. of distilled water, and adds drop by drop concentrated nitric acid until gas is freely given off, which generally happens when from 3 to 4 per cent. of acid have been added. If the decalcification is not complete at the end of twelve hours the objects should be removed to fresh distilled water, and the operation repeated. This process is said to give better results than the various processes of slow decalcification.

818. Eyes of Pecten and other Forms, see Patten, in *Mitth. zool. Stat. Neapel*, vi, 4, 1886, p. 733.

819. Shell.—Sections of non-decalcified shell are easily obtained by the usual methods of grinding, or, which is often a better plan, by the methods of v. Koch or Ehrenbaum, §§ 311, 312. For sections of decalcified shell, Moseley, who has had great experience of this kind of work, particularly recommends the method of decalcification given above, § 817.

820. Injection of Acephala (Flemming, *Arch. f. mik. Anat.*, 1878, p. 252).—To kill the animals freeze them in a salt-and-ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and in a fit state for injection. Chloroform and ether are useless (but see § 16). The injection-pipe may be tied in the heart ; but when this has been accomplished there remains the problem of occluding cut vessels that it is impossible to tie. To this end, after the pipe has been tied, the entire animal is filled and covered up with plaster of Paris. As soon as the plaster has hardened the injection may be proceeded with.

821. Maceration Methods for Mollusca.—For the study of ciliated epithelium the following methods are recommended by Engelmann (*Pflüger's Arch.*, xxiii, 1880, p. 505) :

Cyclas cornea (intestine), maceration in osmic acid of 0·2

per cent. (after having warmed the animal for a short time to 45° to 50° C.). Also, concentrated boracic acid solution.

The Intra-cellular Processes of the Cilia.—The entire intra-cellular fibre apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e. g.* Anodonta) in either bichromate of potash of 4 per cent., or salt solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e. g.* 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic acid solution (5 parts cold saturated aqueous solution to 1 part water).

Bela Haller's **Mixture**, see § 543.

Brock's **Medium**, § 538.

Möbius's **Media**, § 539; the second of these is much recommended by Droost (*Morphol. Jahrb.*, xii, 2, 1886, p. 163) for *Cardium* and *Mya*.

See also the media recommended by Patten (*Mitth. Zool. Stat. Neapel*, vi, 4, 1886, p. 736). Sulphuric acid, 40 drops to 50 grammes of water, is here recommended as a most valuable macerating and preservative agent. Entire molluscs, without the shell, may be kept in it for months.

Arthropoda.

822. General Methods for Arthropoda.—It may safely be stated that, as general methods for the study of chitinous structures, the methods worked out by Paul Mayer (see §§ 4 and 5, and also 57 and 172) are superior to all others. It is absolutely necessary that all processes of fixation, washing, and staining should be done with fluids possessing great penetrating power. Hence picric acid combinations should in general be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid is the most generally useful fixative, 70 per cent. alcohol is the most useful strength for washing out, and tincture of cochineal in alcohol of 70 per cent. (§ 172) is a very generally useful staining fluid. Mayer's hæmacalcium (§ 182) may sometimes be preferable, and alcoholic carmine and borax-carmine will occasionally give more satisfactory results.

Alcoholic picro-sulphuric acid may be indicated for fixing in some cases.

Some forms are very satisfactorily fixed with sublimate. Such are the Copepoda and the larvæ of Decapoda. It is sometimes indicated to use the sublimate in *alcoholic* solution. Some Copepoda, however (*Copilia*, *Sapphirina*), are better preserved by means of weak osmic acid, and so are the Ostracoda. In many cases the osmic acid will produce a sufficient differentiation of the tissues, so that further staining may be dispensed with; *Copilia* and *Phyllosoma* are examples of forms that may be prepared in this simple manner. The pyrogallic process (§ 233) may often prove helpful in the study of such forms.

823. Methods for Clearing and Softening Chitin.—The employment of eau de Javelle or eau de Labarraque, as suggested by Looss, for making chitin transparent and permeable to reagents has been described above, § 557.

List (*Zeit. f. wiss. Mik.*, 1886, p. 212) has obtained good results with Coccidæ by treating them (after hardening) for eighteen to twenty-four hours with eau de Javelle, diluted with four volumes of water. After washing out with water, the objects may be dehydrated with alcohol and imbedded in paraffin, the chitin being sufficiently softened to allow of their being penetrated and good sections being obtained. You may stain before imbedding, with alum-carmine or picro-carmine (five to six days).

The same methods are applicable to the preparation of the ova of Insecta—for instance, *Periplaneta* (see Morgan, *Am. Mon. Mic. Journ.*, ix, 1888, p. 234).

824. Other Depigmentation Methods.—Besides the depigmentation processes discussed in Chap. XXIV, the following methods are available.

Sazepin's **Method for Antennæ of Chilognatha** (*Mém. Acad. Imp. St. Pétersb.*, xxxii, 9, 1884, pp. 11, 12).—Sazepin treated antennæ that have been dehydrated with alcohol by steeping them in chloroform. The reaction is slow, the chitin becomes gradually less opaque, but the pigment does not entirely disappear. In order to remove the last trace of it, it will be sufficient if a drop of fuming nitric acid be now added to the chloroform. The mixture must be occa-

sionally agitated, in order to prevent the acid from floating on the chloroform. The reaction is complete in twenty-four hours.

Employed in this manner, nitric acid has no injurious action on tissues.

825. Eyes of Arthropods.—Lankester and Bourne (*Quart. Journ. Mic. Sci.*, 1883, p. 180) prepared the eyes of *Limulus* as follows :—Alcohol, turpentine, paraffin; sections made and carefully depigmented under the microscope with nitric acid of 5 to 10 per cent., then mounted in balsam, some after staining with borax-carmine, others unstained. Non-depigmented sections also mounted in the same manner.

Hickson (*ibid.*, 1885, p. 243) prepared the eye of a fly as follows :—Remove the posterior wall of the head, and expose the rest, with the eyes *in situ*, for twenty minutes to vapour of osmium. Wash for a few minutes in 60 per cent. alcohol. Harden in absolute alcohol. Make sections. To depigment them, mount them on a slide with Mayer's albumen, remove the paraffin with turpentine, treat them with absolute alcohol, and invert the slide over a capsule containing 90 per cent. alcohol to which a few drops of strong nitric acid have been added. Nitrous vapours are freely given off, and the pigment dissolves. The reaction may be stopped at any moment by washing with pure alcohol.

For dissociation preparations, put the eye or the optic nerve for twenty-four hours into 5 per cent. solution of chloral hydrate, tease, and mount in glycerin. If the elements of the teased tissues be fixed to the slide by means of Mayer's albumen, they may be washed with alcohol and stained *in situ*, or they may be depigmented before staining.

The methods of Parker for eyes of scorpions have been given, § 579.

For the eye of *Homarus* he gives the following staining method (*Bull. Mus. Comp. Zool., Cambridge, U.S.A.*, xx, 1890, p. 1; *Zeit. f. wiss. Mik.*, viii, 1, p. 82). Paraffin sections fixed to slide with Schällibaum's collodion, and passed through alcohol into water, are treated for half a minute with 0·1 per cent. caustic potash solution, well washed with water, and stained for about three hours in Weigert's hæmatoxylin solution at a temperature of 50° C.; washed in water, de-

hydrated, and mounted in balsam. No differentiation of the stain is necessary.

See also the somewhat similar methods of VIALLANES (*Ann. d. Sci. Nat.*, xiii, 1892, p. 354; *Journ. Roy. Mic. Soc.*, 1893, p. 260).

826. Nerve and Muscle of Arctiscoida (DOYÈRE, *Arch. f. mik. Anat.*, 1865, p. 105).—A score or so of *Milnesium tardigradum* are collected (it is well to have a large number, as the process by no means succeeds with all individuals) and put into a test-tube with water that has been deprived of its air by boiling. A drop of oil is run on to the surface of the water, so as thoroughly to exclude the air. After twenty-four to forty-eight hours the animals will be found, not dead, but fixed and extended in a cataleptic state; the circulation of the perivisceral fluid has ceased, the pigment of the cuticle has disappeared or collected into patches that are no hindrance to observation, the entire animal has gained in transparency, and the nervous and muscular systems stand boldly out. The animals are examined in boiled water, unless it be wished to study the phenomena of resuscitation, in which case spring water should be used.

827. Sarcolemma of Insecta (THANHOFFER, *ibid.*, 1882, p. 27).—In order to demonstrate the two plates of the sarcolemma, digest muscle (of an insect) either in the stomach of a living animal (by wrapping it in gauze and introducing it through a fistula) or in artificial gastric juice (in the former case several hours, in the latter half to one hour, at the temperature of the room in summer). Examine in gastric juice.

828. Phalangida (RÖSSLER, *Zeit. f. wiss. Zool.*, xxxvi, 1882, p. 672).—The animals are killed in boiling water; the water is allowed to boil up several times, so that the albumen of the tissues may be coagulated; they are then brought into alcohol, first of 70, then 90 per cent., then absolute, until all water is removed from them. They are then imbedded in soap. The soap is remelted and allowed to cool once or twice, in order to get the objects thoroughly penetrated. Sections are then made, and stained on the slide with some colouring matter dissolved in absolute alcohol.

Paraffin was tried for imbedding, but gave no good results,

on account of the brittleness of the tissues, caused by the preliminary treatment with turpentine or oil of cloves. I fancy that this difficulty would be easily overcome by clearing with cedar oil instead of clove oil, as I have constantly recommended for the special purpose of avoiding brittleness in tissues.

829. Macrotoma plumbea (Sommer, *Inaug. Diss.*, 1884, p. 4; *Zeit. f. wiss. Mik.*, 1885, p. 234).—Fix with boiling water according to the method of Rössler, last section, and harden for several hours in picro-sulphuric acid diluted with 5 volumes of water. Penetrate with chloroform and imbed in paraffin.

830. Aphidæ (see *ante*, § 621).

831. Other Methods for Arthropoda.—For Embryological methods see Chap. XXV, §§ 616—627.

For Spermatological methods see Chap. XXVI, § 645.

Vermes.

832. Cestodes.—This group must of course be chiefly studied by the usual section methods. It is only necessary here to remind the reader that, as pointed out by Vogt and Yung (*Trait. d'Anat. comp. prat.*, p. 204), the observation of the living animal may be of service, especially in the study of the excretory system. And, as shown by Pintner, tæniæ may be preserved alive for several days in common water to which a little white of egg has been added.

Lönnberg (*Centralb. f. Bakteriol. u. Parasitenk.*, xi, 1892, p. 89; *Journ. Roy. Mic. Soc.*, 1892, p. 281) has kept *Triænophorus nodulosus*, a parasite of the pike, alive for a month in a slightly acid pepsin-peptone solution containing from 3 to 4 per cent. of nutritive matter, and less than 1 per cent. of NaCl.

The methylen blue *intra vitam* staining method should be found useful with this group.

833. Trematodes (Fischer, *Zeit. f. wiss. Zool.*, 1884, p. 1).—*Opisthotrema cochleare* may be mounted entire in balsam, after treatment with absolute alcohol, picro-carmine, or hæmatoxylin or ammonia-carmine, and clearing with clove oil. For sectioning, Fischer recommends imbedding in a mass made by dissolving 15 parts of soap in 17·5 parts of 96 per

cent. alcohol. This mass melts at about 60° C., penetrates very rapidly, and solidifies very quickly. The sections should be studied in glycerin.

Wright and Macallum (*Journ. of Morph.*, i, 1887, p. 1) find that *Sphyranura* is for most purposes best fixed in liquid of Flemming, and stained with alum-cochineal.

Cercariæ.—Schwarze (*Zeit. f. wiss. Zool.*, xliii, 1886, p. 45) found that the only fixing agent that would preserve the histological detail of these forms was cold saturated sublimate solution warmed to 35°—40° C.

834. Turbellaria.—Methylen blue will in some cases be found useful for the study of living specimens.

Fixing is difficult, and generally unsatisfactory.

For *Rhabdocœla* Braun (*Zeit. f. wiss. Mik.*, iii, 1886, p. 398) proceeds as follows:—For preparing entire animals, the specimens are got on to a slide, lightly flattened out with a cover, and killed by running under the cover a mixture of three parts of liquid of Lang with 1 per cent. osmic acid solution. Other fixing media than that described were not satisfactory. (Böhmig, however, commenting on this, says that for some of the tissues, such as muscle and body parenchyma, nitric acid and picro-sulphuric acid are very useful.) Sections may be made by the usual paraffin method.

Delage (*Arch. de Zool. exp. et gén.*, iv, 2, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 239) strongly recommends fixation (of Rhabdocœla Acœla) by the osmium-carmine mixture, § 167. Concentrated solution of sulphate of iron is also an excellent fixing medium. The animals (*Convoluta*) die in it fully extended. Liquid of Lang was not successful.

For staining, he recommends either the osmium-carmine stain or impregnation with gold ($\frac{1}{3}$ formic acid, two minutes; 1 per cent. gold chloride, ten minutes; 2 per cent. formic acid, two or three days in the dark. It is well to allow an excessive reduction to take place, and then lighten the stain by means of 1 per cent. solution of cyanide of potassium).

Böhmig, commenting on the above, says that he has obtained very instructive images with Plagiostomidæ fixed with sublimate and stained with osmium-carmine.

Von Graff (*Die Organisation d. Turbellaria Acœla*, Leipzig, 1891; see *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 76) has the following remarks:—Chromo-aceto-osmic acid, followed by hæmatoxylin, is good for the skin; but even this method will not afford a satisfactory preservation of the Rhabdites, which in Acœla and Alloiocœla seem to be destroyed by swelling, whilst

in terrestrial and fresh-water Planaria, Polyclada, and most Rhabdocœla they are better preserved. The same method is also good for the parenchyma of *Amphichœrus cinereus, Convoluta paradoxa,* and *C. sordida.* Sublimate is not good for these forms, but it is good for *Convoluta Roscoffensis.* For some forms it is important to avoid picro-carmine, which destroys the central parenchyma. The nervous system may be investigated by the methods of DELAGE.

For Dendrocœla sublimate solutions, sometimes hot, appear indicated for fixing. CHICHKOFF (*Arch. de Biol.*, xii, 1892, p. 438; *Journ. Roy. Mic. Soc.*, 1893, p. 262) recommends the following for fresh-water Dendrocœla :—2 per cent. sublimate solution, 6 parts; 15 per cent. acetic acid, 4 parts; pure nitric acid, 2 parts; 14 per cent. chloride of sodium, 8 parts; and 2 per cent. alum, 1 part. The animals are said to die in it without contraction. Note also the mixtures of LANG, § 48. The staining method of that author with picro-carmine and borax-carmine (*Fauna u. Flora d. Golfes von Neapel* [*Polycladidea*], 1884, p. 30) seems to be now somewhat antiquated. Mayer's tincture of cochineal, § 172, may be found useful for the study of glands, for which purpose the Ehrlich-Biondi stain should also be employed.

835. Nemertina.—After considerable experience of this difficult group I have to say that I know of no method of fixation that will certainly give good results. My best results have always been obtained with cold saturated sublimate solution, acidified with acetic acid. I have tried most of the energetically hardening fixing agents, such as the osmic and chromic mixtures, and do not recommend them for this group, for they seem (the chromic mixtures and perchloride of iron in particular) to act as irritants, and provoke such violent muscular contractions that the whole of the tissues are crushed out of shape by them. And, besides, they do not kill as quickly as sublimate.

I have found it a good plan to decapitate the animals (in the larger forms), cut them up quickly into lengths (not too long), and throw these sharply into the sublimate, the muscular contractions being less energetic in segments that are no longer in connection with the cerebral ganglia.

Perhaps a better method than this will be found in the

simple process, suggested to me by Prof. DU PLESSIS, of fixing with hot (almost boiling) water. On the few occasions on which I have tried it the animals have died in extension, without vomiting their proboscis; and I think it is certainly worth trial, especially for the larger forms.

I have tried FOETTINGER's chloral hydrate method (§ 14). My specimens died fairly extended, but vomited their proboscides. According to S. LO BIANCO narcotisation with a solution of 0·1 to 0·2 per cent. in sea water is found successful at Naples. For the smaller forms Brocher's monobromated camphor, § 811, may be found useful.

DE CASTELLARNAU (*Estacion Zool. de Napoles*, p. 137) says that Nemerteans can be successfully narcotised by Eisig's alcohol method, described § 13, and I think the process may be a good one for some of the larger forms.

DENDY (see *Journ. Roy. Mic. Soc.*, 1893, p. 116) has succeeded with *Geonemertes* by exposing it for half a minute to the vapour of chloroform.

Intra vitam staining with methylen blue will be found useful in some cases. For the application of the methylen-blue method to the study of the nervous system see BÜRGER, in *Mitth. Zool. Stat. Neapel*, x, 1891, p. 206.

For staining fixed specimens *in toto* I hold that it is well-nigh necessary to employ alcoholic stains, for even the most delicate species are not satisfactorily penetrated by watery stains in any reasonable lapse of time. Borax-carmine or Mayer's alcoholic-carmine may be recommended; not so cochineal or hæmatoxylin stains, on account of the energy with which they are held by the mucin which in general exists in such great abundance in the skin of these animals.

Sections by the paraffin method, after penetration with oil of cedar (chloroform will fail to penetrate sometimes after the lapse of weeks).

836. Nematodes.—The extremely impermeable cuticle of these animals is a great obstacle to preparation. According to Looss (*Zool. Anz.*, 1885, p. 318) this difficulty may be overcome by treating the animals (or their ova, which are in the same case) with eau de Javelle or eau de Labarraque, in the manner described in § 557.

For fixing, most recent authors recommend sublimate

solutions; chromic solutions seem to have a tendency to make the worms brittle.

Staining is frequently difficult, and sometimes alcoholic carmine, § 170, is the only thing that will give fair results.

Braun (see *Journ. Roy. Mic. Soc.*, 1885, p. 897) recommends that small unstained Nematodes be mounted in a mixture of 20 parts gelatin, 100 parts glycerin, 120 parts water, and 2 parts carbolic acid, which is melted at the moment of using. Canada balsam, curiously enough, is sometimes found to make Nematodes opaque.

837. Acanthocephali.—It is very difficult to kill Echinorhynci so as to have the animals duly extended and the tissues well preserved. Neither corrosive sublimate nor strong osmic acid will, as a rule, attain this end, even after preliminary intoxication with tobacco smoke or chloroform, the animal thus treated dying contracted.

Hamann, however (*Jen. Zeit. f. Naturw.*, xxv, 1890, p. 113; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 209), has succeeded with sublimate, and also with alcohol containing a little platinum chloride.

Saefftigen (*Morphol. Jahrb.*, x, 1884, p. 120; *Journ. Roy. Mic. Soc.* [N.S.], v, 1885, p. 147) obtained the best results by killing gradually with 0·1 per cent. osmic acid; the animals placed in this contract during the first hours, but stretch out again and die fully extended.

Another method of killing is treatment with 0·1 per cent. chromic acid; Echinorhynci live for days in it, but eventually die fully extended.

Keiser's methods, see §§ 41 and 61.

838. Gephyrea.—Vogt and Yung (*Anat. comp. prat.*, p. 373) direct that *Sipunculus nudus* be kept for some days in perfectly clean basins of sea water, in order that the intestine of the animals may be got free from sand, which would be an obstacle to section cutting, and then anæsthetised with chloroform, under which treatment they die extended, and may be fixed as desired.

Ward (*Bull. Mus. Comp. Zool.*, Cambridge, *Harvard Coll.*, xxi, 3, p. 144) found the best plan was to put the animals into a shallow dish with sea water and pour 5 per cent. alcohol in a thin film on to the surface of the water. After

four to eight hours, if the animals make no contractions on being stimulated, they may be removed to 50 per cent. alcohol.

S. Lo Bianco says killing with 0·5 per cent. chromic acid or with 0·1 per cent. chloral hydrate in sea water may be tried, but either method is uncertain. *Phascolosoma* and *Phoronis* should be treated by the alcohol method.

Apel (*Zeit. f. wiss. Zool.*, xlii, 1885, p. 461) says that *Priapulus* and *Halicryptus* can only be satisfactorily killed by heat. The animals may either be put into a vessel with sea water and be heated on a water-bath to 40° C.; or they may be thrown as rapidly as possibly into boiling water, which paralyses them so that they can be quickly cut open and thrown into one third per cent. chromic acid, or picro-sulphuric acid.

839. Rotatoria.—By far the most important method for the study of this group consists in the observation of the living animals. Great difficulty exists in the way of getting them to keep sufficiently quiet. Vogt and Yung (*Anat. comp. prat.*, p. 420) say that a drop of solution of any of the soluble salts of strychnin run under the cover sometimes renders service. Weber (*Arch. de Biol.*, viii, 4, 1888, p. 713) finds that strychnin, prussic acid, and curare act too strongly; of all the reagents he tried, 2 per cent. solution of hydrochlorate of cocaïn gave the best results. Warm water gave him good results for large species, such as those of *Hydatina* and *Brachionus*.

Hardy (*Journ. Roy. Mic. Soc.*, 1889, p. 475) recommends thick syrup added drop by drop to the water. Hudson (*ibid.*, p. 476) mentions weak solution of salicylic acid.

Hofer's hydroxylamin method has been given, § 16, and Tullberg's chloride of magnesium method, § 17. Brocher's monobromated camphor, § 811, should be tried; as should also the processes of Eismond and of Jansen, § 858. Methylen blue, § 114, may be found useful.

Permanent preservation of Rotifers has hitherto been considered by those who have tried it to be well-nigh impossible. Rousselet (*Journ. Quek. Mic. Club*, v, 2, 1893, p. 205; *Journ. Roy. Mic. Soc.*, 1893, p. 262) has, however, lately had a considerable measure of success. The animals should be

narcotised by means of cocain of 1 to 2 per cent. strength added to the water in a trough containing them. They should be watched under the microscope until the cilia have just ceased to vibrate, and should then be fixed by running strong solution of Flemming down the sides of the trough by means of a pipette. They are left for fifteen minutes, washed in five or six changes of distilled water, and permanently mounted in distilled water containing a trace of solution of Flemming (about 8 drops to the ounce). For minutiæ concerning the different species see the original.

Annelida.

840. Cleansing Intestine of *Lumbricus* (Kükenthal, *Journ. Roy. Mic. Soc.*, 1888, p. 1044).—Put the animals into a tall glass vessel which has been filled up with bits of moistened blotting-paper. They gradually evacuate the earthy particles from the gut, and fill it instead with paper.

Vogt and Yung (*Traité d'Anat. Comp. Prat.*, v) recommend coffee-grounds instead of paper; paper becomes rather hard when imbedded, whereas coffee-grounds cut fairly well.

841. *Lumbricus* may be anæsthetised by putting the animals into water with a few drops of chloroform. Perrier has pointed out that it is better not to let the chloroform act directly in solution on the animals, but to put them into water in a shallow dish, set up a watch-glass with chloroform in the corner of it, and cover the whole. In half an hour the worms will be more or less narcotised, and if allowed to remain will die in a state of extension.

Cerfontaine (*Arch. de Biol.*, x, 1890, p. 327; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 210) much recommends curare, administered by interstitial injection of a dose of about 2 c.c. of a 1 : 500 solution. The animal should afterwards be put into water, and after a quarter of an hour will be found dead.

In order to kill *Criodrilus lacuum*, Collin (*Zeit. f. wiss. Zool.*, xlvi, 1888, p. 474) puts the animals into a closed vessel with a little water, and hangs up in it a strip of blotting-paper soaked in chloroform. Kükenthal (*Die mik. Technik*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 61) puts Annelids into a glass cylinder filled with water to the height of 10 centimetres, and then pours 70 per cent. alcohol to a depth of 1 to 2 centi-

metres on to the water. The animals will be found sufficiently narcotised for fixation in from four to eight hours. For *Opheliadæ* he also employs 0·1 per cent. of chloral hydrate in sea water.

Many marine Chætopoda may be successfully narcotised (S. Lo Bianco) in sea water containing 5 per cent. of alcohol, or by means of the mixture, § 12.

The *Polychæta sedentaria* offer the difficulty of a complex and very contractile branchial apparatus. They may sometimes be satisfactorily fixed by bringing them rapidly into corrosive sublimate. Cold, not hot solutions should be taken, as heat frequently shrivels up the branchiæ. The species of *Polychæta errantia* that offer a contractile branchial apparatus, as *Eunice* and *Onuphis*, may be treated in the same way.

S. Lo Bianco advises killing Chætopteridæ, Sternaspidæ, *Spirographis*, *Protula*, by putting them for half an hour into 1 per cent. chromic acid. I have satisfied myself that good show specimens can be obtained in this way; but I doubt the histological preservation of the parts being so good as with sublimate specimens. Some of the *sedentaria* may be got protruded from their tubes by leaving them for some hours in 0·1 per cent. chloral hydrate in sea water (S. Lo Bianco).

See also the methods §§ 14 to 19, and § 811.

I can recommend as a good fixing and hardening mixture for Annelids in general the following fluid, due to Ehlers (I do not know whether it has been published elsewhere):— To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The proportion of acetic acid indicated is sufficient to counteract any tendency to shrinkage due to the chromic acid.

842. Blood-vessels of Annelids (Kükenthal, *Zeit. f. wiss. Mik.*, 1886, p. 61).—The animals should be laid open and put for two or three hours into *aqua regia* (4 parts of nitric acid to 2 of hydrochloric acid). The ramifications of the vessels will then be found to be stained black, the rest of the preparation yellow.

843. Nerves of Annelids.—The methylen-blue method and the bichromate of silver method of Golgi (the *rapid* method). For the latter see v. Lenhossék (*Arch. f. mik. Anat.*, xxxix, p. 102; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 342).

844. Hirudinea.—For the methods of killing see those given for Lumbricus in § 841, also §§ 14 to 19, and § 811.

WHITMAN (*Meth. in mic. Anat.*, p. 27) recommends that they be killed with sublimate. This reagent kills leeches with such rapidity that they die in general without having time to change the attitude in which they were found at the moment when the liquid came into contact with them.

Injection.—WHITMAN (*Amer. Natural.*, 1886, p. 318) states that very perfect natural injections may often be obtained from leeches that have been hardened in weak chromic acid or other chromic liquid. He considers that these injections are the best for the purpose of the study of the circulatory system by means of sections.

Of course Hirudinea (or any other Annelids) on which it is desired to make artificial injections must be killed by some procedure that leaves the tissues in a state that will allow the injection to run freely.

JAQUET (*Mitth. Zool. Stat. Neapel*, 1885, p. 298) advises that leeches be put into water with a very small quantity of chloroform; they soon fall to the bottom of the vessel and remain motionless. They should be allowed to remain a day or two in the water before injecting them.

Echinodermata.

845. Holothurioidea.—These animals are difficult to fix on account of their contracting with such violence under the influence of irritating reagents as to expel their viscera through the oral or cloacal aperture. It has been recommended that they be seized by the middle of the body and firmly squeezed in the hand, and so plunged in a fixing liquid (acetic acid, for instance), or that they be anæsthetised (in the case of *Synapta* and *Cucumaria*) by adding ether to the water in which they are contained. So far as my experience goes, I am bound to say that I know no better way of killing them than that of simply putting them into fresh water, in which they generally die without contraction, and with their tentacles extended. Of course the histological preservation of the parts is detestable.

S. LO BIANCO puts Holothurids into pure sea water until they have expanded their tentacles, then seizes them with

forceps behind the tentacles, so as to mechanically render impossible their withdrawal, and immerses the anterior part of the body in acetic acid, whilst at the same time an assistant injects 90 per cent. alcohol through the anus.

Vogt and Yung (*Anat. Comp. Prat.*, p. 641) say that *Cucumaria Planci* (*C. doliolum*, Marenzeller) is free from the vice of expelling its intestines under irritation; but they recommend that it be killed with fresh water, or by slow intoxication with alcohol, chromic acid, or sublimate added to the sea water in which it is contained.

Synapta may be allowed to die in a mixture of equal parts of sea water and ether or chloroform (S. Lo Bianco).

846. Asteroidea.—There are great difficulties in the way of fixation here, too. It is quite possible to obtain a fixation of the ambulacral feet, branchiæ, and tentacles in the extended state, by throwing the animals into boiling water, and then bringing them into a fixing liquid. But this method has the fault that the fixing liquid so employed only penetrates extremely slowly into the interior of the animal, and therefore does not give a good fixation of internal organs.

Hamann (*Beitr. z. Hist. d. Echinodermen*, ii, 1885, p. 2) finds it preferable to inject the living animal with a fixing liquid. The cannula should be introduced under the integument at the extremity of a ray, and the liquid injected into the body-cavity. The ambulacral feet and the branchiæ are soon distended by the fluid, and as soon as it seems to have penetrated sufficiently the animal is thrown into a quantity of the same reagent.

The study of the *eyes* presents points of special difficulty. In order to study them in sections, with the pigment preserved *in situ*, the eye should be removed by dissection, should be hardened in a mixture of equal parts of 1 per cent. osmic acid and 1 per cent. acetic acid, and imbedded in a glycerin gum mass, or some other mass that does not necessitate treatment with alcohol (which dissolves out the pigment, leaving the pigmented cells perfectly hyaline). For maceration use one-third alcohol, the aceto-osmic mixture failing to preserve the rods of the pigmented cells.

847. Ophiuridea.—Should be killed in fresh water if it be

desired to avoid rupture of the rays (De Castellarnau, *La Est. Zool. de Napoles*, p. 135).

848. Larvæ of Echinodermata.—I am greatly obliged to my able friend Dr. Barrois for kindly writing down for me (for the *Traité des Méth. techn.*, from which they are translated) the following instructions, which are the outcome of a prolonged and minute study of the metamorphoses of the Echinodermata.

Pluteus.—In order to a fruitful study of the metamorphoses of the Echinoidea and Ophiuridea it is necessary to obtain preparations that offer the advantages presented by the study of the living larvæ; and especially such as give distinct images of the different organs, and show the calcareous skeleton preserved intact (a point of considerable importance, since this skeleton frequently affords landmarks of the greatest value). These preparations should further possess the following points:—They should give clear views of the region of formation of the young Echinoderm (which is generally opaque in the living larva). And they should possess sufficient stiffness to allow of the larva being turned about in any desired way, and placed in any position under the microscope.

It is not very easy to obtain preparations fulfilling these conditions, on account of the difficulty of obtaining a selective stain whilst preserving the integrity of the calcareous skeleton. The following method is recommended:—*Pluteus* larvæ are fixed in a cold saturated solution of corrosive sublimate, in which they remain not more than two or three minutes. They are then washed with water, and brought into dilute Mayer's cochineal (§ 172). This should be so dilute as to possess a barely perceptible tinge of colour. The objects should remain in the stain for from twelve to twenty-four hours, being carefully watched the while, and removed from the stain at the right moment and mounted in balsam, or, which is frequently better, in oil of cloves or cedar-wood. This method is perfectly satisfactory for the study of the chief phases of metamorphosis.

Auricularia and Bipinnaria.—The method described above is equally applicable to these forms, and seems to be altogether the best method for the study of the metamorphosis of Bipinnaria. The earlier stages of the metamorphosis of Auricularia

are better studied by fixing with osmic acid, staining with Beale's carmine, and mounting in glycerin.

Larvæ of Comatula.—The best method for the study of the embryonal development of *Comatula* consists in fixing with liquid of Lang, and staining with dilute borax-carmine. It is important (for preparations that are not destined to be sectioned) to use only *dilute* borax-carmine, as the strong solution produces an over-stain that cannot easily be reduced.

Narcotisation by chloral hydrate before fixing is useful, especially for the study of *Pentacrinus* larvæ and of the young *Synaptæ* formed from Auricularia. Without this precaution you generally get preparations of larvæ either shut up (*Pentacrinus*), or entirely deformed by contraction (young *Synaptæ*).

See also MacBride on the development of *Amphiura squamata, Quart. Journ. Mic. Sci.*, xxxiv, 1892, p. 131; *Journ. Roy. Mic. Soc.*, 1893, p. 117 (osmic acid followed by liquid of Müller and alcohol; decalcification with nitric acid in alcohol; staining with Mayer's paracarmine or hæmalum).

849. Anthozoa.—*Narcotisation.*—For suitable narcotisation methods see §§ 9 to 19, and § 811.

Fixation.—In *Le Attinie, Fauna u. Flora d. Golfes v. Neapel* the following hints are given:—Hot corrosive sublimate often gives good results. In the case of the larger forms the solution should be injected into the gastric cavity, and a further quantity of the liquid be poured over the animals.

Freezing sometimes gives good results. A vessel containing Actiniæ is put into a recipient containing an ice-and-salt freezing mixture and surrounded by cotton wool. After freezing, the block of ice containing the animals is thawed in alcohol or some other fixing liquid.

The Zoantharia with calcareous skeletons are difficult to deal with on account of the great contractility of the polyps. Sublimate solution, which ought very often to be taken boiling, sometimes gives good results. De Castellarnau (*La Est. Zool. de Napoles*, p. 132) says that this process succeeds well with *Dendrophyllia, Antipathes, Astroides, Cladocora,* and *Caryophyllia.*

The Alcyonaria have also extremely contractile polyps. In the last edition I suggested for their fixation either hot

sublimate solution or glacial acetic acid (§ 53). S. Lo Bianco has since recommended essentially similar processes. Garbini (*Manuale*, p. 151) says that the polyps may be fixed in the state of extension by drenching them with ether, and then bringing them into strong alcohol.

Wilson (*Mitth. Zool. Stat. Neapel*, 1884, p. 3) kills Alcyonaria with a mixture of 1 part of strong acetic acid and 2 parts of concentrated solution of corrosive sublimate, the animals being removed as soon as dead and hardened for two or three hours in concentrated sublimate solution.

Braun (*Zool. Anz.*, 1886, p. 458) recommends that for both Zoantharia and Alcyonaria a little osmic acid be added to the sublimate employed for fixation. For *Alcyonium palmatum*, *Sympodium coralloides*, *Gorgonia verrucosa*, *Caryophyllia cyathus*, and *Palythoa axinellæ* he proceeds as follows:—The animals are left for a day or two in a glass vessel, so that the polyps may become thoroughly extended. They are then suddenly drenched with a mixture of 20 to 25 c.c. of concentrated solution of sublimate in sea water with four to five drops of 1 per cent. osmic acid. This is allowed to act for five minutes.

(This method also gives good results with *Hydra* and some Bryozoa and Rotifers.)

Schultze (*Biol. Centralb.*, 1887, p. 760) says that for Pennatulidæ with large polyps the gradual addition of fresh water is a good plan.

Sections.—For preparing sections, besides the usual methods for sectioning decalcified specimens, we have the valuable methods of von Koch and Ehrenbaum, §§ 311 and 312, which, being applicable to undecalcified specimens and furnishing preparations showing at one and the same time soft parts and hard parts *in situ*, render most inestimable services.

Maceration.—For the Hertwigs' well-known method (*Jen. Zeit.*, 1879, p. 457) see § 542. The tissues should be left to macerate in the acetic acid for at least a day, and may then be teased in glycerin.

List (*Zeit. f. wiss. Mik.*, iv. 2, 1887, p. 211) recommends dilute liquid of Flemming. Tentacles of *Anthea cereus* and *Sagartia parasitica* treated for ten minutes with a mixture of 100 c.c. of sea-water with 30 c.c. of Flemming's liquid (the strong solution, § 36), then washed out for two or three hours in 0·2 per cent. acetic acid, and teased in dilute glycerin, give

fine dissociations of the connective, sensory, and urticant cells of the ectoderm, and after removal of the epidermis allow of the demonstration of ganglion-cells and the supporting lamella. Picro-carmine may be used for staining.

850. Hydroidea, Polypoid Forms.—For suitable *narcotisation* methods see those quoted in last section.

Fixation.—In general the polyps may be very well killed in saturated sublimate solution, in which they should be plunged for an instant merely, and be brought into alcohol. The solution should be employed cold in general for Gymnoblastea, hot for most Calyptoblastea.

Ether attentively administered gives good results with Campanularidæ. *Hydra* is very easily killed by treatment with a drop of osmic acid on a slide. Breckenfeld (*Amer. Mon. Mic. Journ.*, 1884, p. 49) obtains good results by heating the animals in a drop of water on a slide for from three to five seconds over a petroleum lamp. The methods for sections are the usual ones.

The *methylen-blue* method of *intra vitam* staining is applicable to this group, see, *inter alias*, Zoja, l. c., § 114.

851. Medusæ: Fixation.—There is some difficulty in properly fixing the forms with contractile tentacles, which easily roll up on contact with reagents. The best results I have had with these forms have been obtained by means of van Beneden's acetic acid method, § 53, followed by alcohol. A similar method, with the difference that a mixture of chromic acid and alcohol is used for washing out instead of pure alcohol, is recommended by De Castellarnau (*La Est. Zool. de Napoles*, p. 133) for *Oceania, Lizzia, Bougainvillia, Podocoryne, Syncoryne*, &c. The secret of success with the long-tentacled forms lies in a trick of manipulation. Put sufficient acetic acid into a deepish dish, hold it in your left hand (or, better, in both hands if you have an assistant), and keep it moving in a circle so as to communicate a vortex motion to the liquid. Take up a medusa in a spoon with as little sea-water as possible, and throw it into the moving liquid, and keep the liquid steadily swirling round so as to cause the tentacles to trail out at full length behind the animal until it is thoroughly fixed, then pass carefully into alcohol. Do not, unless you are very expert, try to fix more than one medusa at a time;

it is also better to keep the specimens separate, even in the alcohol, as, if several are together, it generally happens that their tentacles become entangled. The method is due to S. Lo Bianco. *Oceania conica* and *Tiara* may usefully, according to S. Lo Bianco, be narcotised with 3 per cent. alcohol in sea water before fixation. Liquid of Kleinenberg, which I have seen much used for the fixation of these and similar forms, is, in my opinion, histologically a very objectionable reagent for the purpose.

Trachymedusæ and Acalephæ may be fixed in the usual way in chromic or osmic mixtures. Osmic acid may conveniently in some cases be added to the sea water containing the animals, which should be removed to fresh water as soon as they begin to turn brown.

852. Medusæ: Sections.— I am not acquainted with any perfectly satisfactory method of sectioning these extremely watery organisms. Paraffin and collodion will afford good sections of some organs, but are certainly not satisfactory as all-round methods for this group. Some modification of the method employed by the Hertwigs (*Nervensystem der Medusen*, 1878, p. 5) might be successful. They imbedded in liver with the aid of glycerin gum, and hardened the objects and the mass in alcohol. I should think better results would be obtained by one of the freezing methods given in §§ 315 to 320.

853. Medusæ: Maceration.—The methods of the Hertwigs, § 542, have deservedly become classical for the study of the tissues of this group.

Amongst other advantages of this process it should be noted that the reduction of osmic acid by albuminates is greatly hastened by the presence of acetic acid, which in the case of animals so transparent and poor in cells as Medusæ is an advantage for the study of the nervous system; for ganglion-cells and nerve-fibrils reduce osmium quicker than common epithelium-cells. They become greenish brown, and are easily distinguished from surrounding tissues. Doubtless in many cases the pyrogallic acid reaction, § 233, would give better results.

The isolation of the elements of the macerated tissues is

best done by gently tapping the cover-glass (which may be supported on wax feet). This gives far better results than teasing with needles. A camel-hair pencil also sometimes renders good service.

854. Siphonophora.—This group contains some of the most difficult forms to preserve that are to be found in tho whole range of the animal kingdom. You have not only to deal with the very great contractility of the zooids, but with the tendency to general disarticulation of the swimming-bells and prehensile polyps.

The cupric sulphate method of BEDOT, § 44, is practised as follows:—Bedot directs that a large quantity of 15 to 20 per cent. solution of the salt be suddenly added to the sea water containing the animals. As soon as they are fixed (which happens in a few minutes) a few drops of nitric acid are to be added and mixed in (this is in order to prevent the formation of precipitates), and the whole is left for four to five hours. The specimens are then to be hardened *before* bringing them into alcohol. Bedot recommends that this be done with solution of Flemming. The strong solution is the one that should be taken, and it should be *added* to the solution of sulphate containing the Siphonophore, about two volumes of it being taken for one of the sulphate solution. The whole should be left for at least twenty-four hours. After hardening in the mixture a few drops of 25 per cent. alcohol should be added to the fluid with a pipette, being dropped in as far as possible from the colony, which should be disturbed as little as possible; and further alcohol, of gradually increasing strength, should be added so gradually that the strength of 70 per cent. be not attained under fifteen days at least. Ninety per cent. alcohol should be used for definite preservation.

I have tested this method. I do not find that the histological preservation is superior to that obtained by means of the usual processes; but the method is certainly a valuable one in so far as *it enables one to preserve specimens with all their swimming-bells and polyps in situ,* a result which is not obtained by means of the usual methods.

The cupric and zinc sulphate mixture of FRIEDLANDER has been given, § 44.

S. Lo Bianco employs for the majority of Siphonophora a mixture of 10 c.c. of saturated solution of corrosive sublimate with 100 c.c. of 10 per cent. solution of copper sulphate. This is used as in Bedot's process. *Diphyes, Rhizophysa,* and *Physalia,* however, are killed with sublimate solutions; *Velella* with chromo-picric acid, or a mixture of 100 c.c. of sublimate solution with 50 c.c. of 1 per cent. chromic acid; *Porpita* by poisoning with liquid of Kleinenberg.

Korotneff's method of paralysing with chloroform has been given in § 11. I would only add that I have seen *Physophora* very successfully killed by the careful administration of ether.

855. Ctenophora: Fixation.—The small forms are very easily prepared by means of osmic acid. The large forms are for the most part extremely difficult to deal with on account of the extraordinary delicacy of the tissues. S. Lo Bianco employs for *Callianira* a mixture of 1 part 0·5 per cent. chromic acid, 1 part concentrated pyroligneous acid, and 2 parts concentrated sublimate solution. For *Beroe Forskalii* he recommends the cupric sulphate mixture, last section (I would point out that the cupric sulphate method is very unsuitable for many forms, as it makes them opaque, and so heavy that they easily break). The majority of forms he treats with a mixture of 100 parts of 1 per cent. chromic acid and 2 parts 1 per cent. osmic acid, *Cestus Veneris* with chromo-acetic acid, rolling it up like a watch-spring in order that it may not become deformed by its own weight.

Samassa has succeeded in making sections of Ctenophora by means of the double-imbedding method, § 306 (see *Arch. f. mik. Anat.,* xl, 1892, p. 157; *Zeit. f. wiss. Mik.,* 1893, p. 340).

Porifera.

856. Spongiæ: Fixation.—The smaller forms (Calcispongiæ) can be fairly well fixed by the usual reagents, osmic acid being one of the best. For the larger forms no satisfactory fixing agent has yet been discovered, so far as I can ascertain. The tissues of this group are very watery, very delicate, very friable after hardening, and macerate with the greatest facility. For all but very small specimens, absolute alcohol

is apparently the best fixing agent. If any watery fluid be preferred, care should at all events be taken to get the sponges into strong alcohol as soon as possible after fixation, on account of the rapidity with which maceration sets in in watery fluids. FIEDLER (*Zeit. f. wiss. Zool.*, xlvii, 1888, p. 87) has been using (for *Spongilla*), besides absolute alcohol, an alcoholic sublimate solution, and the liquids of Kleinenberg and Flemming, with good effect.

Staining.—On account of the great tendency to maceration above referred to, I hold (notwithstanding many recommendations of watery stains that are to be found in the literature of the subject) that alcoholic stains be alone employed for staining sponges, and I particularly recommend Mayer's tincture of cochineal as giving the best results personally known to me.

Sectioning.—Calcareous sponges may be decalcified in alcohol slightly acidified with hydrochloric acid, and then imbedded in the usual way. Siliceous sponges may be desilicified by Mayer's hydrofluoric acid method mentioned *ante*, § 574. But in view of the really dangerous nature of this operation, I feel bound to recommend that it be avoided. Fair sections may be obtained from sponge tissues well imbedded in paraffin without previous removal of the spicula. The spicula appear to be cut; probably they break very sharply when touched by the knife. Of course you will not use your best knives for cutting such sections.

Preparation of Hard Parts.—Siliceous spicules are easily cleaned for mounting by treating them on a slide with hot concentrated nitric or hydrochloric acid, or solution of potash or soda. The acids mentioned are very efficient, but it must be pointed out that they will attack the silex of some delicate spicules. Thus DEZSÖ found that the small stellate spicules of the cortex of *Tethya lyncurium* are completely dissolved by boiling hydrochloric acid. Potash solution is therefore frequently to be preferred, notwithstanding that, in my experience, it does not give such clean preparations.

According to NOLL, eau de Javelle is preferable to any of these reagents (see § 556).

Impregnation with Silver (see § 217).

Larvæ of Spongiæ.—SCHULTZE (*Zeit. f. wiss. Zool.*, xxxi, p. 295) places the ova and larvæ of *Sycandra raphanus* in

hanging-drop moist chambers, oxygenated by means of a few fronds of green algæ. He also (*ibid.*, xxxiv, 1880, p. 416) found that the best sections of the more advanced *sessile* larvæ of *Plakina* were obtained by selecting larvæ that had settled down on thin fronds of algæ, and treating them, together with the fronds, with osmic acid, staining with alum-carmine, and bringing into paraffin in the usual way.

Protozoa.

857. Introductory.—Since the Protozoa may be considered as free cells, and their peculiar organs known as "nucleus" and "nucleolus," "macronucleus" and "micronucleus," &c., present in the main the same reactions as cell-nuclei, it is evident that the reagents and methods of cytology are in great part applicable to this group. One of the most generally useful of these reagents will be found in the acid solution of *methyl green;* it is the reagent that allows of the readiest and best demonstration of the presence and form of the nucleus and nucleolus (Balbiani et Henneguy, *Compt. rend. Soc. de Biol.*, 1881, p. 131). The Ehrlich-Biondi mixture, § 259, will of course in many cases be indicated.

Amongst useful reagents not mentioned in the following descriptions of the methods employed by different authors, I call attention to the weak solutions of alum, potash, and borax, which serve to demonstrate the striations of the cuticle and the insertions of the cilia of Infusoria.

858. Methods for quieting Infusoria.—The narcotisation methods, §§ 14 to 18, are available for this purpose, as is also the monobromated camphor process of Brocher, § 811, which I believe has been found very successful with Vorticellidæ.

According to Schürmayer (*Jen. Zeit.*, xxiv, 1890, pp. 402—470; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 493) nitrate of strychnin in weak solution, 0·01 per cent. or less, gives good results with some forms, amongst which are *Stentor* and *Carchesium*. Antipyrin in concentrated solution (0·1 per cent.) or cocain of 0·01 per cent. seems only to have given good results as regards the extension of the stalk in stalked forms.

Eismond (*Zool. Anz.*, No. 352, Dec., 1890, p. 723) has proposed a mechanical means of slowing the movements of small organisms (small worms and crustacea as well as

Ciliata). He directs that a drop of thick aqueous solution of cherry-tree gum be added to the water containing the organisms (gum arabic and the like, it is stated, will not do). The objects remain fixed in their places, with cilia actively moving, and all vital processes retaining their full activity. I am greatly obliged to Dr. GRÜBLER for having been at much pains in making inquiry for me, concerning the cherry-tree gum that should be used. It appears that this gum is a somewhat insoluble one, and it is difficult to get hold of a sample that will give a good solution. Further, the solutions will not keep, and must be made up fresh every day. In the face of these difficulties it would seem that the method is at present a far from perfect one. It should, however, be stated that CERTES (*Bull. Soc. Zool. France*, xvi, 1891, p. 93; *Journ. Roy. Mic. Soc.*, 1891, p. 828) has found that the method gives excellent results. He has also found that an *intra vitam* stain may be obtained by adding methyl blue or "violet dahlia No. 170," to the gum solution.

A similar process of inhibiting movements whilst preserving life has been worked out by JENSEN (after STAHL; see *Biol. Centralb.*, xii, 1892, 18, 19, p. 556; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 483; *Journ. Roy. Mic. Soc.*, 1892, p. 891). A solution of 3 grms. of gelatin in 100 c.c. of ordinary water is made by the aid of heat. This makes a jelly at the normal temperature. It is slightly warmed, and a drop of it is mixed in a watch-glass with a drop of water containing the organisms. This plan is said to afford great facilities for the vivisection of Infusoria.

858. Staining *intra vitam*.—The possibility of staining Infusoria *intra vitam* was discovered independently and almost simultaneously by BRANDT (*Verh. d. physiol. Ges. Berlin*, 1878), by CERTES (*Soc. Zool.*, 25 janv., 1881), and by HENNEGUY (*Soc. philom.*, 12 fév., 1881).

CERTES found that living Infusoria stain, while continuing in life for a certain time, in weak solutions of cyanin, Bismarck brown, dahlia, violet 5 B, chrysoidin, nigrosin, methylen blue, malachite green, iodine green, and other tar colours, and hæmatoxylin. The solutions should be made with the liquid that constitutes the natural habitat of the organisms. They should

be very weak, that is, of strengths varying between 1 : 10,000 and 1 : 100,000. For cyanin, 1 : 500,000 is strong enough.

The "nucleus" may be stained in the living organism by dahlia and malachite green. Bismarck brown only colours the "nucleus" of certain species (*Nychtoterus, Opalina*—Henneguy). The "nucleus" frequently behaves differently in allied species.

A double stain of the nucleus (green) and protoplasm (violet) may be obtained by the simultaneous employment of dahlia and malachite green.

Examination in a coloured medium in which the organisms do not stain, but show up on a coloured background in a manner that produces somewhat the effect of dark-ground illumination, is sometimes helpful. Certes (*Bull. Soc. Zool. de France,* xiii, 1888, p. 230) recommends solution of anilin black for this purpose; Infusoria will live in it for weeks. Fabre-Domergue (*Ann. de Microgr.,* ii, 1889, p. 545; *Journ. Roy. Mic. Soc.,* 1889, p. 832) recommends concentrated solution of diphenylamin blue.

859. Fixing and Preserving.—Pfitzner (*Morph. Jahrb.,* xi, 1885, p. 454) used concentrated solution of picric acid run in under the cover.

Blanc (*Zool. Anz.,* 1882, p. 22) advises liquid of Kleinenberg diluted with about a volume of water, and acidified with acetic acid.

Korschelt (*Zool. Anz.,* 1882, p. 217) recommends 1 per cent. osmic acid, or for Amœbæ, 2 per cent. chromic acid.

Lansberg (*ibid.,* p. 336) advises the same reagents, but recommends bringing the organisms into the fixing liquid with a pipette, instead of running in the fixing liquid under the cover.

Cattaneo's **Methods** (*Bollettino Scientifico,* iii and iv; *Journ. Roy. Mic. Soc.,* 1885, p. 538) are as follows:—Fix for a few minutes with $\frac{1}{3}$ per cent. aqueous solution of chloride of palladium. This is the best fixing agent, as it hardens in a few minutes without blackening the structures. Double chloride of gold and cadmium also fixes well, and brings out nuclei even better than the palladium. Solution of iodide of mercury and potash ($\frac{1}{2}$ per cent.) is useful for bringing out protoplasmic networks, as it stains the granules of protoplasm

black. Corrosive sublimate in 5 per cent. solution gives good results. Osmic acid causes darkness and opacity in the preparations. Nitrate of silver (in ½ to 1 per cent.) solution may be used, the objects being washed out with solution of acid sulphate of soda. Chromic, picric, and picro-sulphuric acids and bichromate of potash are only second-rate fixing agents for this purpose.

Brass (*Zeit. f. wiss. Mik.*, i, 1884, p. 39) employs for fixing unicellular organisms the following liquid:

Chromic acid	1 part.
Platinum chloride	1 „
Acetic acid	1 „
Water	400 to 1000 parts.

For protozoa that are opaque through accumulation of nutritive material, he proceeds as follows:—The organisms are treated for three or four minutes with liquid of Kleinenberg, and then for some time with boiling water. They are then brought into water containing a small proportion of ammonia, in which they reassume their natural forms and dimensions. The ammonia is then neutralised by addition of a little acetic acid, and the preparation is stained with borax-carmine or ammonia-carmine. After washing, the objects are mounted in dilute glycerin. This treatment is said to afford extremely transparent preparations.

Brass also obtained good results with sublimate solution.

Certes (*Comptes rend.*, 1879, 1 sem., p. 433) makes permanent preparations as follows:—Fix with osmic acid of 2 per cent. (In the case of very contractile Infusoria, place a drop of the solution on the cover-glass, and place it on the drop of water that contains them. But generally speaking it is best to employ only the vapour of the solution, exposing the organisms to its action for not more than from ten to thirty minutes.)

The objects having been covered, the excess of liquid is removed by means of blotting-paper, and the following stain is allowed to flow in:

Glycerin	1 part.
Water	1 „
Picro-carmine	1 „

(Eosin may also be used. Soluble anilin-blue does not give such good results.) The stain should be placed at the edge

of the cover, and the slide put away in a moist chamber, in order that the water may evaporate very slowly and be changed very gradually for the glycerin mixture; if this precaution is not taken, shrinkage may occur. When the exchange has taken place, strong glycerin may be added, and gradually substituted for the dilute glycerin.

Certes states that the organisms thus prepared are fixed perfectly in their natural form, and allow of the study of the minutest detail of cilia, flagella, and the like, with the highest powers; the green coloration of Euglenæ and Paramecia is preserved. The nulear structures are sharply brought out by the picro-carmine.

Saville Kent and Berthold (*Manual of the Infusoria; Journ. Roy. Mic. Soc.*, 1883, p. 451) prefer a brownish-yellow solution of potassium iodide to osmic acid for fixing.

The employment of *vapour* of iodine has been described, § 66.

Du Plessis (Vogt et Yung, *Trait. Anat. Comp. Prat.*, p. 92) recommends fixation with 0·2 per cent. solution of corrosive sublimate. Let the preparation *dry up*, and if the organisms have preserved their shape, stain and mount in balsam.

Fol (*Lehrb.*, p. 102) fixes delicate marine Infusoria (*Tintinnodea*) with the perchloride of iron solution, § 52, added to the water containing them, and stains with gallic acid as directed § 231, and states that this is the only method that has given him good results, especially as regards the preservation of cilia.

Künstler (*Journ. de Microgr.*, 1886, pp. 17 and 58).—For Monadina. Fixation by means of a drop of very concentrated osmic acid solution (a grm. of osmium dissolved in only a few c.c. of distilled water).

Longhi (*Bull. Mus. Zool. Univ. Genova*, 4, 1892; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 483) has obtained good results by means of sulphate of eserin. A 1 : 10 per cent. solution is taken, and to each 10 c.c. of it one drop of 1 per cent. sublimate solution is added.

See also the methods of Fabre-Domergue, *Ann. de Microgr.*, ii, 1889, p. 545, and of Schewiakoff, *Biblioth. Zool.*, v, 1889, p. 5; *Journ. Roy. Mic. Soc.*, 1889, pp. 832, 833; Zoja, *Boll. Sci. Pavia*, 1892; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 485.

860. Demonstration of Cilia (Waddington, *Journ. Roy. Mic. Soc.*, 1883, p. 185).—Solution of tannin, or a trace of alcoholic solution of sulphurous acid.

861. Stains for Flagella.—The celebrated method of Löffler has run through several forms (*Centralb. f. Bacteriol.*, vi, 1889, p. 209; vii, 1890, p. 625; *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 359; vii, 3, 1890, p. 368; *Journ. Roy. Mic. Soc.*, 1889, p. 711; 1890, p. 678), of which that given here is the latest. To 10 c.c. of a 20 per cent. solution of tannin are added 5 c.c. of cold saturated solution of ferrous sulphate and 1 c.c. of (either aqueous or alcoholic) solution of fuchsin, methyl violet, or "Wollschwarz." The mixture will require for some forms the addition of a few drops of 1 per cent. solution of caustic soda; *e. g.* for typhoid bacilli, 1 c.c.; for *Bacillus subtilis*, 28 to 30 drops; for bacilli of malignant œdema, 36 to 37 drops. Some other forms will require besides the addition of a trace of sulphuric acid to the soda solution, so for cholera bacteria, half a drop to 1 drop; for *Spirillum rubrum*, 9 drops.

Cover-glass preparations are made and fixed in a flame in the usual way, special care being taken not to over-heat. Whilst still warm the preparation is treated with mordant (*i. e.* the above-described mixture), and is heated for half a minute until the liquid begins to vaporise, after which it is washed in distilled water and then in alcohol. It is then treated in a similar manner with the stain, which consists of a saturated solution of fuchsin in anilin water, the solution being preferably neutralised to the point of precipitation by cautious addition of 0·1 per cent. soda solution.

Trenkmann (*Centralb.*, vi, 1889, p, 433; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 79) mordants for several hours at the normal temperature in a 1 per cent. solution of tannin in 0·5 per cent. hydrochloric acid, and stains for several hours in carbolic fuchsin; and gives also two other similar methods.

Brown (*The Observer*, iii, 1892, p. 298; *Journ. Roy. Mic. Soc.*, 1893, p. 268) mordants for several hours in a mixture of 30 gr. tannin, 12 drops anilin oil, and 1 fl. oz. of alcohol, which may, if required, be alkalised by addition of a trace of caustic soda (so for *Spirillum undula* and *Bacillus ulna*), or may be acidified for others with a little hydrochloric acid. The cover is stained by the process of heating over a flame for a few minutes with any anilin-water solution of fuchsin, methyl violet, dahlia, methyl green, &c., neutralised with caustic soda as in Loeffler's process, or with a solution of rosanilin in anilin water.

APPENDIX.

862. The Usual Alcohols.

Strength.	Made approximately as under.	Observations.
Absolute	See § 63. The so-called "absolute alcohol" of commerce is generally of about 98 per cent. strength.	This grade is *not* necessary for ordinary work.
95 per cent.	This is the average strength of the common strong commercial alcohol, which ranges in general from 94 per cent. to 96 per cent. according to temperature. The strength of this, or of the following, should be determined by means of an areometer (Gay Lussac's being very convenient), so as to form a starting-point for the following mixtures.	This is the usual grade for dehydrating before clearing. It is the highest grade that should be used for dehydrating celloidin sections.
90 per cent.	May be made approximately by taking 100 vols. 95 per cent. alcohol, and 5·5 vols. water.	This is the usual strength of the strongest commercial *Methylated Spirit*, which (if free from mineral naphtha) may be taken instead of pure alcohol for common work. If naphtha be present the alcohol becomes turbid on the addition of water. Oil of bergamot will clear from this grade.

Strength.	Made approximately as under.	Observations.
85 per cent.	Made by taking 100 vols. 90 per cent. alcohol, and 6·5 vols. water.	*Rectified Spirit B.P.* is a little weaker than this, viz. 84·5 per cent.
70 per cent.	May be made of 100 vols. 90 per cent. alcohol or methylated spirit, and 31 vols. water; or 100 vols. 85 per cent. alcohol, and 23 vols. water.	Only exceptionally powerful clearers, such as anilin oil, will clear from this grade; see § 353. This is the proper grade in general for preserving organisms and tissues in (but see p. 3 *sub. fin.*); higher grades should not generally be used unless it is desired to harden. This is the proper grade for washing out borax-carmine stains, corrosive sublimate after fixing, &c.
50 per cent.	Made by taking 100 vols. 90 per cent. alcohol, and 84·7 vols. water; or 100 vols. 70 per cent. alcohol, and 41·7 vols. water.	This is the strength of *Proof Spirit.*
"One-third alcohol"	Made by taking 1 vol. of 90 per cent. alcohol, and 2 vols. water.	See § 63.

863. Histological Reagents and Apparatus.—The great importance of working with trustworthy chemicals has been insisted on in § 94, where also the addresses of some chemists who make a speciality of histological reagents have been given. As regards Dr. GRÜBLER's products, so often quoted in the foregoing pages, I would add that they should either be ordered from him direct (address, *Herrn Dr. G. Grübler,*

Chemiker, 12, Baiersche Strasse, Leipzig, Germany), or, if ordered through any agent, should be ordered to be sent in the original packages, signed *and dated* by him. This is in order to ensure the due *freshness* of the products; many of them will not keep well for very long.

Glass and other apparatus can be obtained as well as chemicals from the above-quoted houses. I would call attention to the varied usefulness of the "Siebdosen" or sieve-dishes of ZIMMERMANN and SUCHANNEK (vide *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 159). They consist of a covered glass capsule into which is fitted a "sieve" made of a watch-glass pierced with holes and supported on legs. It is evident that the arrangement is very handy, not only for staining, washing out, treatment with vapours, &c., but for any operation in which it is desirable to have specimens supported in the upper layers of a quantity of reagent. They are sent out in a very neat form by Dr. Grübler.

Want of space compels me to suppress the lists of suggested reagents given in the last edition under the headings "The Laboratory Table" and "The Zoologist's Travelling Case." Either collection may still be obtained from Dr. Grübler, the latter in appropriate bottles, fitted into a case measuring 1 foot 4 inches × 5½ inches × 4½ inches, at the price of about £2 5*s.*, or a case somewhat larger, yet not too heavy to be carried in the hand, at about £3.

864. Cleaning Slides and Covers.—The readiest way known to me of freeing slides from balsam, damar, and cement is to wet with water and scrape with an old knife, using afterwards, if necessary, one of the solvents mentioned below.

HANAMAN, *Journ. Roy. Mic. Soc.*, i, 1878, p. 295; *American Naturalist*, xii, p. 573.—To a cold saturated solution of bichromate of potash add ⅛ of its bulk of strong sulphuric acid (care must be taken on account of the heat and vapours evolved).

HENEAGE GIBBES, *ibid.*, iii, 1880, p. 392.—Place the cover-glasses in strong sulphuric acid for an hour or two, wash well until the drainings give no acid reaction; wash first with methylated spirit, and then with absolute alcohol, and wipe carefully with an old silk handkerchief.

SEILER, *ibid.*, p. 508.—*New* slides and covers are placed for a few hours in the following solution:

Bichromate of potash	2 ounces.
Sulphuric acid	3 fluid ounces.
Water	25 „

Wash with water. The slides may be simply drained dry; the covers may be wiped dry with a linen rag.

Slides and covers that have been used for mounting either with balsam or a watery medium are treated as follows:—The covers are pushed into a mixture of equal parts of alcohol and hydrochloric acid, and after a few days are put into the bichromate solution and treated like new ones. The slides

are scraped free of the mounting medium with a knife and put directly into the bichromate solution.

Fol (*Lehrb.*, p. 132) recommends either a solution containing 3 parts of bichromate, 3 of sulphuric acid, and 40 of water; or simply dilute nitric acid.

Garbini (*Manuale*, p. 31) puts slides for a day into 10 per cent. sulphuric acid, then washes, first with water and then with alcohol.

Behrens (*Zeit. f. wiss. Mik.*, 1885, p. 55) treats slides first with concentrated nitric acid, then with water, alcohol, and ether.

James (*Journ. Roy. Mic. Soc.*, 1886, p. 548) treats used slides with a mixture of equal parts of benzin, spirit of turpentine, and alcohol.

Knauer (*Centralbl. f. Bakt.*, x, 1891, p. 8; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 187; *Journ. Roy. Mic. Soc.*, 1891, p. 833) recommends boiling for twenty or thirty minutes in 10 per cent. lysol solution, then rinsing with cold tap water till clear.

Nias (*Journ.*, pag. cit.) finds it is sufficient to boil with washing soda, and rinse.

865. Gum Mucilage for Labels, &c.—The *Journ. of the Chemical Soc.* says that the adhesive qualities of gum may be very much exalted by the addition of aluminium sulphate (the so-called "patent" alum) to the mucilage. "2 grms. of crystallised aluminium sulphate, dissolved in 20 grms. of water, is added to 250 grms. strong gum arabic solution (2 grms. in 5 grms. water). Ordinary solutions of gum arabic, however concentrated, fail in their adhesive power in many cases, such as the joining together of wood, glass, or porcelain; prepared, however, according to the above receipt, the solution meets all requirements" (from *Public Opinion*, Feb. 19th, 1886).

Fol (*Lehrb.*, p. 148) advises that slides be prepared for labelling by spreading over one end a layer of aluminium-chloride gelatin dissolved in acetic acid, and allowing it to dry before putting on the label.

Why do not the glass makers furnish slides with roughened (ground) end-surfaces for the reception of labels?

For four other receipts for gums and pastes for labels, see Eliel, in *Engl. Mechan.*, 1887, p. 535; *Amer. Mon. Mic. Journ.*, 1887, p. 93; *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 69.

Vosseler (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 459) recommends, for attaching protective cardboard ridges to slides, a syrup-thick solution of bleached shellac in alcohol.

866. Green Light.—The suggestion of the employment of green light in microscopy is, I believe, due to Englemann (*Pflüger's Arch.*, 1880, p. 550). He strongly recommends the use of green light for delicate observations, as giving sharper definition, allowing finer detail to be seen, and tiring the eyes less than white light. Green glass of sufficiently good quality is found in commerce. The glass is best put between the mirror and the object, *e. g.* on the diaphragm. Blue glass (cobalt or ammonio-sulphate of copper) is also useful, but less so than green. Red light is most hurtful. "The explanation of these points, so important in practice, may be found in the results obtained by Lamansky in his researches on the 'Limits of Sensi-

bility of the Eye to the Different Colours of the Spectrum' (*Arch. f. Ophthalm.*, xvii, p. 123, 1871)." I would add that for lamp-light work, especially fine work with high powers, either green or blue glass is, according to my experience, a *sine quâ non* if the best attainable results be desired. I always use blue *cover-glasses*, putting from one to four of them on the diaphragm of the condenser. For some unexplained reason I find I get better results by means of several superposed thin glasses than by one thick one. Spectral blue glass can be obtained from Mr. Pillischer, the optician, in Bond Street.

867. Object-holder to Cambridge Microtome.—Since § 271 was written the Cambridge Sci. Inst. Co. have brought out an adjustable object-holder to their rocking microtome, fulfilling the required conditions as regards facility of orientation.

868. Obregia's Serial Section Method.—This method, which was originally described in the *Neurologisches Centralb.* for 1890, and is given in the third edition of WOODHEAD'S *Practical Pathology*, had escaped my attention. It has been lately recommended for class purposes, as being very safe and convenient, by GULLAND (*Journ. of Path.*, February, 1893). Slides, or glass plates of any size, are coated with a solution made of—

Syrupy solution of powdered candy-sugar made with boiling distilled water	30 c.c.
Absolute alcohol	20 „
Transparent syrupy solution of pure dextrin made with distilled water .	10 „

They are dried slowly for two or three days until the surface is just sticky to the moist finger. Sections are arranged and heated for a few minutes to a temperature slightly above the melting-point of the paraffin. The paraffin is removed by some solvent, such as xylol or naphtha, and this is in turn removed by alcohol. The alcohol is poured off, and the sections are covered with solution of celloidin or with a solution of 3 per cent. of photoxylin in a mixture of equal parts of ether and absolute alcohol. The plates are left to evaporate in a horizontal position, and when the sections are required the sheet of collodion is cut into ribbons, which are floated off in water, and further treated as desired, *e. g.* as in Weigert's process, § 340. (It is well to divide the sheet of collodion into ribbons by running the point of a knife

down it as soon as evaporation has produced a very slight solidification, and the evaporation must not be artificially hastened.)

869. The Water and Albumen Section-fixing Method.—A careful and detailed description of the method shortly described after HENNEGUY (§ 328) is given by OHLMACHER (*Journ. Amer. Med. Ass.*, April, 1893), who has independently worked out the same process.

870. Monobromated Camphor for Narcotisation. — Since writing the note calling attention to this new method in § 811 I have learnt that the method is *not* applicable to marine organisms, as the camphor is not sufficiently soluble in salt water. But for the most diverse fresh-water forms it has proved highly convenient.

871. Gilson's Mercuro-nitric Fixing Mixture.—I regret to find that by an unaccountable oversight I have omitted to mention this liquid, the formula of which has been several times published. I am indebted to Prof. GILSON for kindly sending me the latest formula, which is as follows:

Nitric acid of 46° strength (this would be sp. gr. 1·456, or 80 per cent., nearly)	78 c.c.
Glacial acetic acid	22 „
Corrosive sublimate . . .	95 grms.
60 per cent. alcohol . . .	500 c.c.
Distilled water	4400 „

When required for marine animals add a few crystals of iodine, which will prevent the formation of precipitates of sea salts. If in any case the preparations should show a granular precipitate, due probably to an abundance of phosphates in the tissues, the precipitate may be removed by washing with water containing a little tincture of iodine (*not* iodide of potassium, which would precipitate the sublimate).

This mixture affords in general a faithful and delicate fixation, and gives to tissues an excellent consistency. Objects may remain in it for a considerable time without hurt. Tissues are left in a state very favorable for staining. The liquid has a high degree of penetration. A treatment for a few days with it will serve to remove the albumen from the ova of Batrachians.

872. GILSON'S **Picro-alum-Carmine** (§ 157).—Professor Gilson writes me that the formula given in § 157 is erroneous as containing too much picric acid, and has been good enough to send me an amended receipt, with the remark, however, that all these formulæ seem to have lost their *raison d'être* by the publication of Mayer's carminic acid stains. As that is really quite the opinion I had come to myself on the matter, I suppress the new formula, merely making use of this opportunity to reiterate the opinion that for the present Mayer's stains are victorious all along the line.

873. Chloral Hydrate Jelly (kindly communicated by Prof. GILSON).—1 vol. of gelatin, melted *secundum artem*, and 1 vol. of Price's glycerin. Mix, and add 1 vol. of chloral hydrate (*i. e.* add crystals of chloral until the volume of the mixture has increased by one half); warm till dissolved. This gives a very highly refractive aqueous mounting medium, which is found useful for opaque tissues that it is desired not to dehydrate.

A similar medium is published by GEOFFROY, *Journ. de Botan.*, 1893, p. 55 (see *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 476). He dissolves, by the aid of as little heat as possible, 3 to 4 grms. of gelatin in 100 c.c. of 10 per cent. aqueous solution of chloral hydrate.

874. Sulphurous Acid as a Bleaching Agent.—Prof. GILSON writes me that alcoholic solution of sulphurous anhydride (SO_2) is very convenient for the rapid decoloration of bichromate objects. A few drops suffice.

875. ZACHARIAS'S **Carmine Stain.**—Notwithstanding the remarks on carmine stains in general just made (§ 872), it seems worth while to call attention to a process due to ZACHARIAS (*Verh. d. Vers. D. Naturf. u. Aerzte, Bremen*, 1891, p. 121; recommended by SCHÜTZ, *Monatschr. f. prakt. Dermat.*, xiv, 1892, p. 397; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 476), as it involves a somewhat new principle. Tissues, after fixation in any of the usual media, "Flemming" included, are stained for half an hour to an hour in aceto-carmine (§ 158), are well washed in water, and brought for five hours into 1 per cent. solution of sulphate of protoxide of iron. After thorough washing they are dehydrated and mounted in balsam. Karyokinetic figures dark black, the ground of a delicate neutral tone.

876. Rapid Modification of Weigert's Stain.—The following is given by KAISER in *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 468:—Sections treated from three to five minutes with 1 per cent. solution of bichromate of potash, washed in Weigert's hæmatoxylin fluid (§ 688), and warmed over a flame in a fresh quantity of the stain until bubbles are formed, rinsed with water, and differentiated by the method of Pal, § 692 (sulphite of sodium being, however, taken instead of the potassium salt). As will be seen on

reference to § 696, this modification of Weigert's process is essentially identical with that of LISSAUER.

Another process given by KAISER, loc. cit., is as follows:—Sections to be mordanted for a few minutes in a mixture of 1 part liquor ferri sesquichlorati, 1 part distilled water, and 3 parts rectified spirit, then stained in Weigert's stain changed several times until a heavy precipitate is formed, then washed in water and differentiated as before. They are then to be rinsed in ammoniated water and stained in a 0·1 per cent. solution of fuchsin in rectified spirit, or in a solution of 1 part of naphthylamin brown in 100 of spirit and 200 of water. The fuchsin will stain in about half a minute, the naphthylamin brown in from three to five minutes. Mount as usual. Medullated nerves blue, non-medullated red or brown. The reaction will be enhanced if the sections be warmed in the stain.

877. Preservation of Golgi's Bichromate of Silver Preparations. —KALLIUS (*Anat. Hefte*, ii, 1892, p. 269; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 477) has worked out the following process. Take 20 c.c. commercial hydroquinone developing solution and 230 c.c. distilled water (the hydroquinone solution may be made up with 5 grms. hydroquinone, 40 grms. sodium sulphite, 75 grms. carbonate of potassium, and 250 grms. distilled water). At the instant of using, further dilute the solution with one third to one half its volume of absolute alcohol, and put the sections into it for several minutes; they become dark grey to black. In order to ascertain whether reduction is complete, throw a section into a solution of hyposulphite of soda (about 10 parts to 50 of water): chromate of silver will quickly dissolve, whilst metallic silver will not be attacked. As soon as reduction is complete the sections are put for ten to fifteen minutes into 70 per cent. alcohol, then brought for five minutes into the above-given solution of hyposulphite of soda, and thence into a large quantity of distilled water, where they should remain for twenty-four hours or more. Lastly, dehydrate in the usual way, and mount under a cover. All the details which in the undeveloped preparations were brown are now black on a light ground. After-staining with carmine, &c., may be employed. Other developers were tried, and gave satisfactory reductions, but they caused a red or brownish discoloration of the preparations.

To the references to the paper of HUBER given in § 712, add that to *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 479. The reader will of course understand that the balsam is to be

heated on the slide, with the section in it, until it immediately sets hard on cooling.

878. Methylen Blue for Impregnation Purposes.—APÁTHY has a note in *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 466, to the effect that the best methylen blue for impregnation—in fact, the only one that will give *exactly* the results described by him (see § 118)—is that obtained from E. MERCK, of Darmstadt, and quoted in his price list as "*medicinisches* Methylenblau," and described on the label as "Anilin-blau, Methylen, chemisch rein und chlorzinkfrei."

879. Mounting Methyl Green Stains.—Attention was called in § 109 to the difficulty of mounting methyl green stains in balsam on account of the imperfect resistance of the colour to alcohol. SQUIRE says, in his *Methods and Formulæ*, p. 36, that a thorough washing with water previous to the treatment with alcohol has the effect of fixing the colour in the nuclei, so that it is less easily removed by the spirit.

880. GOODALL'S Rapid Method for Preparing Spinal Cord (*Brit. Med. Journ.*, May, 1893, p. 947; *Journ. Roy. Mic. Soc.*, 1893, p. 405).—Cut sections of fresh tissue with a freezing microtome; float them on to water, and as soon as possible drain and float them on to pyridin. After a quarter of an hour wash in water; stain with 0·25 per cent. aqueous solution of anilin blue-black, followed by picro-carmine; dehydrate and clear in pyridin; mount in balsam thinned with pyridin. See also *ante*, § 90.

881. Demonstration of Living Trichinæ (BARNES, *Amer. Mon. Mic. Journ.*, xiv, 1893, p. 104; *Journ. Roy. Mic. Soc.*, 1893, p. 406).—A piece of trichinised muscle of the size of a pea should be placed in a bottle in a mixture of 3 gr. of pepsin, 2 dr. of water, and 2 minims of hydrochloric acid. The whole should be kept at body temperature for about three hours with occasional shaking. The flesh and cysts being dissolved, the fluid is poured into a conical glass, and allowed to settle; the trichinæ are drawn off from the bottom with a pipette, got on to a slide with water, and examined on a hot stage.

INDEX.

The numbers refer to the Paragraphs, not to the Pages.

A.

Abbe, mounting medium, 415.
Absolute alcohol, uses and preparation, 2, 63, 355, 862.
Acanthocephali, 49, 60, 837.
Acephala, 813, 820, 821; and *see* Mollusca.
Acetate of alumina, for mounting, 373.
Acetate of copper, 64, 386.
Acetate of lead, 87, 732.
Acetate of potash, 374, 394, 529.
Acetate of uranium, 65.
Acetic acid for fixing, 53, 54, 811, 849 851.
Aceto-carmine, 158, 875.
Acetone, for dehydration, 2.
Achromatic figure, 639.
Acid anilin dyes, 788.
Acid, free, test for in tissues, 132.
Acid fuchsin, 95, 98, 131; for nerve tissue, 722, 772, 773.
Acidophilous cell-elements, 788, 793.
Acids, *see* Acetic, Chromic, Formic, Hydrochloric, &c.
Acidulated alcohol, 63.
Actiniaria, 9, 10—14, 17, 19, 542, 849.
Adamkiewics, stain for nerve-centres, 774.
Adipose tissue, 36, 787.
Agar-agar, section fixative, 332.
Agassiz and Whitman, pelagic ova, 610.
Albumen, imbedding masses, 314, 321; injection mass, 498; section-fixing process, 328; removal of, from ova, 598 *et seq.*, 871.
Alcohol, its action on tissues, 2; fixing, 63; absolute, 63; "one third," 63; acetic, 54; acidulated, 63; picric, 62; for hardening, 88, 89; macerating, 525; preserving tissues, 2, 378; clearing, 355; table of the usual grades, 862.
Alcohol-balsam, 419.
Alcoholic carmines, 168—171.
Alcoholic cochineal, 172, 173.
Alcoholic hæmateïn stains, 182—184, 190, 194.
Alcyonaria, 7, 849.
Alcyonella, 14.
Alcyonium, 849.
Alferow, silver impregnation, 213.
Alizarin, 200, 756.
Allen, osmium impregnation, 233.
Alt, Congo red, nerve stain, 724.
Altmann, fixing methods, 37, 637; bioblasts, 634, 637, 641; impregnation and corrosion methods, 558.
Alum, for fixing, 45; preserving, 372; solubility in alcohol, 184; ammonia-alum, solubility in water, 185; roche-alum, 190.
Alum-carmine, 153—157; ditto, with acetic acid, 156; with osmic acid, 155; with picric acid, 157.
Alum-cochineal, 146, 154.
Alumina, acetate, 373.
Amaroecium, 612.
Amber varnish, 446, 447.
Ammonia, bichromate, 80, 732, 737, 738; neutral chromate, 81.
Ammonia-alum, 185.
Ammonia-carmine, 163—165, 745.

The numbers refer to the Paragraphs, not to the Pages.

Ammoniated hæmatoxylin, 188*a*.
Ammonio-nitrate of silver, 213.
Ammonium, molybdate, for impregnation, 234; for fixing nuclei, 637; sulphocyanide, 530; vanadate, 511.
Amphibia, for cytological study, 633; embryology, 82, 598 *et seq.*
Amphioxus, 662.
Amphipoda, embryology, 626.
Amphiura, 848.
Amyl nitrite, 500.
Amyloid matter, 109, 112.
Anchinia, 811.
ANDEER, phloroglucin, 562.
ANDRES, Actiniæ, 10, 11, 12, 849; section-stretcher, 280.
ANDREWS, imbedding apparatus, 273.
ANGELUCCI, retina, 682.
Anilin, for clearing, 305.
Anilin dyes, generalities, 92, 95; classification into acid and basic, 788; classified list, 95; Hermann-Böttcher or Flemming staining process, 95—100, 258.
Anilin black, 93, 95, 130, 757.
Anilin blue, 95, 128, 756.
Anilin blue-black, 125, 757.
Anilin green, 95, 100, 105, 142, 808.
Anilin oil for clearing, 305, 353.
Anilin red, 107.
Anilin violet, 111.
Anilin water, 101, 275, 305.
Aniseed imbedding mass, 322.
Annelida, 14, 840—844; embryology, 630.
Anodonta, 16.
Anthea, 849.
Anthozoa, 849.
Antipathes, 849.
Antipyrin for narcotisation, 858.
APÁTHY, celloidin imbedding, 299; preserving the blocks, 302; serial sections, 338, 339; cement, 455; hæmatoxylin stain, 194; double-stain for nervous tissue, 785; methylen blue impregnation, 115, 117—119, 878; mounting medium, 400.
APEL, *Sipunculus*, and *Halicryptus*, 838.
Aphides, preparation and embryology, 621.
Aplysia, 813, note.
Appendicularia, 217.
Aqua Javelli, 556, 581, 616.
Aqueous stains recommended, 5.
Aquiferous pores of Molluscs, 813.
Arabin, 329.
Araneina, embryology, 622.
ARCANGELI, carmines, 167.
Archil, 206.
Archiplasm, archoplasm, 639.
Arctiscoida, 826.
Areolar tissue, 786.
ARMANNI, logwood nerve stain, 694.
ARNOLD, critique of Flemming's mixture, 35, 36; hæmatoxylin stain, 192.
ARNSTEIN, methylen blue method, 117, 119.
Arsenic acid for decalcification, 572.
Arthropoda, fixing, 4, 5, 57, 822; general methods, 822; clearing and softening chitin, 823; depigmentation, 823—825; eyes, 825; nerve and muscle, 826; sarcolemma, 827; embryology, 616—627; spermatology, 645.
Artificial fecundation, 584.
Artificial indigo, 125.
Artificial œdemata, 786.
Artificial pigment spots, 77, 731, 732.
Artificial saliva, 531.
Artificial serums, 524, 361, 362.
Ascaris, ova, 54, 639, 644.
Ascidians, 18, 811.
Asphalt varnish, 436.
Asphyxiation as a killing method, 18.
Astacus, embryology, 118, 625.
Asteracanthion, 14.
Asteroidea, 846.
Astroides, 849.
Attraction spheres, 639.
AUBERT, cements, 430, 433.
AUERBACH, stains for male and female chromatin, 645.
Aurantia, 98.
Auréoline, 580.
Auricularia, 848.
Aves, embryology, 589—594.

The numbers refer to the Paragraphs, not to the Pages.

Axis cylinder, 719 *et seq.*; stains, 721 *et seq.*
Axolotl, ova, 599.

B.

Baber, picro-carmine, 162.
Babes, cytological methods, 638, 643; safranin stain, 101; supersaturated do., 638.
Balbiani, "noyau vitellin," 640; living ova, 585; ova of Phalangida, 623; Protozoa, 857; spermatological methods, 645.
Balfour, embryology of Aves, 589, 590; of Araneina, 622.
Ballowitz, muscle of Cephalopoda, 676.
Balsam, 417—419, 421, 428.
Balzer, elastic tissue, 795.
Barff, Boroglyceride, 404.
Barnes, observation of Trichinæ, 881.
Barrett, retina, 682, 685.
Barrois, embryology of Echinodermata, 848.
Basic anilin dyes, 788.
Basophilous cell-elements, 788, 793.
Bastian, gold method, 228.
Batrachia, central nervous system, 742, 784.
Baumgarten, cytological methods, 643; fuchsin and methylen blue stain, 268; picro-borax-carmine, 171; triple stain, 270.
Bayberry tallow, 287.
Bayerl, stain for cartilage, 242, 801; decalcification, 564.
Beale, carmine stain, 165; digestion fluid, 549; glycerin jelly, 408; injections, 501, 503, 504; perservative fluids, 370.
Bechtereff, hardening brain, 732.
Beck, cements, 430.
Becker, microtome, 271.
Beckwith, gold-staining nerve centres, 768.
Bedot, fixing process, 44, 854.
Beevor, Weigert's hæmatoxylin, 691.
Behn, hardening skin, 646.
Behrens, cements, 430, 433; amber varnish, 446; cleaning slides, 864.
Béla Haller, macerating mixture, 543.
Bellonci, preparation of brain, 741.
Bell's cement, 430, 434.
Benczur, staining nerve-centres, 756.
Benda, hæmatoxylin, 639; double-stain for sperm-cells, 639; spermatological method, 645; Weigert's hæmatoxylin, 690.
Beneden, van, acetic acid fixing method, 53, 811; acetic alcohol, 54; Ascidians, 811; corrosive sublimate liquid, 47; cytological methods, 644; embryology of Mammalia, 588, of Tænia, 628.
Beneden, van, and Neyt, cytological methods, 54, 637, 639, 644.
Bengal rose, 95, 136.
Bengalin, 125.
Benzol, for clearing, 354.
Benzo-azurin, 107, 127.
Benzo-purpurin, 95, 133.
Bergamot oil, 347.
Bergonzini, staining mixture for plasma cells, 793.
Berlin blue, 478.
Berlinerblau, F., regeneration of Weigert's hæmatoxylin, 688.
Bernheimer, hæmatoxylin for retina, 683.
Beroe, 855.
Berry's Hard Finish, 424.
Bevan Lewis, *see* Lewis, Bevan.
Bianco, S. Lo, Molluscoida (Bryozoa), 812; Mollusca, 813; Nemertina, 835; Gephyrea, 838; Chætopoda, 841; Holothurioidea, 845; Anthozoa, 849; Medusæ, 851; Siphonophora, 854; Ctenophora, 855; Alcyonaria, 849; Tunicata, 18, 811; narcotising mixture, 12.
Bichloride of mercury, *see* Corrosive sublimate.
Bichromate of ammonia, 80, 732, 737, 738.
Bichromate of potash, for fixing and hardening, 40, 43, 75—79, 731—737, 739; for maceration, 535;

The numbers refer to the Paragraphs, not to the Pages.

bleaching bichromate objects, 75, 874.
Bichromate of silver impregnation, 704 *et seq.*
Bickfalvi, digestion fluid, 550.
Biebricher Scharlach, 95, 134.
Biedermann, methylen blue method, 117, 666; nerve-endings in muscle, 666, 667.
Bilberry juice stain, 209.
Biniodide of mercury medium, 414.
Bioblasts, Altmann's, 634, 637, 641.
Biondi, staining mixture, 259, 639; methods for blood, 803, 806.
Bipinnaria, 848.
Bismark brown, 93, 95, 107, 110, 638.
Bitume de Judée, 436.
Bizzozero, cytological method, 643; gentian violet, 102; picro-carmine, 162; maceration, 646; mucus cells, 807; blood, 804, 805.
Bjeloussow, injection, 499.
Blackburn, vegetable wax, 287.
Black currant stain, 209.
Blackley blue, 125.
Bladder of frog, 677.
Blanc, saffron stain, 204; infusoria, 859.
Blattida, embryology, 619.
Blaue, glandular epithelium, 646.
Bleaching, 575 *et seq.*; bichromate objects, 75, 874; Erlicki objects, 77; osmic objects, 29.
Bleu carmin aqueux, 123a.
Bleu de Lyon, 95, 124.
Bleu de nuit, 124.
Bleu lumière, 95, 123.
Blochmann, ova of Amphibia, 598.
Blood, 802—806.
Blood-vessels of Annelids, 842, 844.
Blue-black, 130.
Bobretsky, embryology of Lepidoptera, 618.
Boccardi, gold method, 228, 670; motor plates, 670.
Böhm, gold method, 228.
Böhmer, hæmatoxylin, 186.
Böhmig, rhabdocœla, 834.
Böhn, carmine, 165.
Boll, gold staining nerve-centres, 767.
Bolsius, picro-alum carmine, 157, 872; do. with hæmatoxylin, 239.
Bone, decalcification, 559 *et seq.*, 797, 798; preparation, 796—801.
Borax-carmine, Grenacher's, 167, 169; Gibbs', 167; Nikiforow's, 167; Woodward's, 167; Baumgarten's, 171; Haug's, 167; with indigo-carmine, 241, 242.
Bordeaux, 98.
Boric-acid carmine, 167.
Born, paraffin imbeding, 273; section fixing, 331; reconstruction of sections, 587.
Boroglyceride, 404.
Böttcher-Hermann staining process, 95—100; p. 60, note.
Bougainvillea, 851.
Bouma, stain for cartilage, 801.
Boveri, ovum of Ascaris, 639, 644; medullated nerve, 720.
Boyer, embryology of Teleostea, 608.
Brachionus, 839.
Brady, chloral hydrate medium, 376.
Brain, *see* Central nervous system and Neurological methods.
Bramwell, brain, 779.
Branca, redwood nerve stain, 694.
Brandt, glycerin jelly, 409; Protozoa, 858.
Brass, alcoholic carmine, 170; bleaching osmic objects, 29; paraffin imbedding, 275, 286, 287; Protozoa, 859.
Braun, Alcyonaria, &c., 849; Rhabdocœla, 834; Nematodes, 836.
Brazil-wood nerve stain, 694.
Breckenfeld, *Hydra*, 850.
Breglia, Weigert's hæmatoxylin, 688, 694.
Bremer, motor plates, 670.
Bride, Mac-, *see* Macbride.
Brocher, narcotisation method, 811, 870.
Brock, macerating medium, 538.
Brösicke, staining method, 233.
Brown, stains for flagella, 861.
Brücke, digestion fluid, 550; injections, 478.
Brun, mounting medium, 109, 402.

The numbers refer to the Paragraphs, not to the Pages.

Brunotti, gelatin imbedding mass, 296.
Brunswick black, 437.
Bryozoa, 7, 14, 15, 812.
Budge, injection, 515.
Bugula, 812.
Bumpus, dry celloidin imbedding, 303.
Burci, elastic tissue, 795.
Burckhardt, nervous system of *Protopterus*, 742.
Bürger, nervous system of Nemertina, 835.
Busch, eosin and hæmatoxylin, 251; decalcification, 559, 560.
Busse, photoxylin imbedding, 297; celloidin do., 299, 301.
Bütschli, paraffin imbedding, 276; hæmatoxylin stains, 185, 196.

C.

Cacao-butter imbedding mass, 287.
Cajal, Ramón y, silver chromate impregnation, 714, 715, 717; terminations of nerves and tracheæ, 669, 714; retina, 683, 684, 715; sympathetic ganglia, 715.
Calberla, methyl green, 109; methyl green and eosin, 260; indulin, 125; glycerin mixture, 405; macerating mixture, 531; imbedding, 314.
Calcispongiæ, 856.
Calcium chloride mounting medium, 375.
Caldwell, serial sections, 327.
Callianira, 855.
Cambridge rocking microtome, 271, 867.
Camphor, monobromated, for narcotisation, 811, 870.
Canada balsam, for mounting, 417—419, 421; imbedding method, 313.
Canfield, iris, 679.
Cannel oil, 346.
Caoutchouc, for section fixing, 336; cement, 435.
Capparelli, dichroïsm of methyl violet, 112.
Carbolic acid, 305, 351, 367.
Carbolised alcohol, 682.
Carbolised syrup, 365.
Carbonic acid narcotisation method, 19.
Carchesium, 858.
Carcinoma corpuscles, 101.
Carmalum, 145, 151.
Carmine, analysis of, 144; generalities on, 144—150; theory of staining with, 144—146; practice of staining, 149; classification of the formulæ, 150; for what purposes useful, 147.
Carmine blue, 123 a.
Carmine double stains, 236—249.
Carmine stains, acetic, 156, 158, 167, 875; alcoholic, 168—173; alum-carmine, 153—157; ammonia-carmine, 163—165; borax-carmine (aqueous), 167 (alcoholic), 169, 171; boric acid, 167, 171; Carmalum, 145, 151; Carminroth, 167; chloride of aluminium, 145, 152; cochineal, 154, 172, 173; hydrochloric acid, 170; lithium, 166; neutral, 164, 165, 167; osmium-carmine, 167; Paracarmine, 168; picro-carmine, 159—162; picro-alum-carmine, 157, 872; putrefied carmine, 165; salicylic acid, 167; soda carmine, 167; uranium carmine, 162, 167; and *see* the names of the respective authors, as also the separate entries, "Alum-carmine," "Borax-carmine," &c.
Carminic acid, 144—146, 148.
Carminroth, 167.
Carnoy, acetic alcohol, 54; chromo-aceto-osmic acid, 36; cement, 450; cytological methods, 635—637, 644; salt solution, 359; tannin solution, 387; spermatology of Arthropods, 645.
Carpenter, preservative media, 378; cements and varnishes, 430.
Carrière, corpuscles of Herbst and Grandry (gold method), 652; eyes of Gastropods, 815; maceration of nervous tissue, 781.

The numbers refer to the Paragraphs, not to the Pages.

CARTER, injection mass, 470.
Cartilage, 801.
Caryophyllia, 849.
CASTELLARNAU, de, Actiniæ, 849; Eucharis, 748; Lamellibranchiata, 813, Medusæ, 851; Nemertina, 835; Ophiuridea, 847; Zoantharia, 849.
Castor oil for mounting, 425.
CATTANEO, corpuscles of Golgi, 673; Infusoria, 52, 859.
Caustic potash and soda, for maceration, 529; for corrosion, 555.
Cedar oil, for minute dissections, 4; for paraffin imbedding, 275; clearing, 99, 344; for mounting, 417, 418.
Cell-granules, 641, 788 *et seq.*
Cell - researches, *see* Cytological methods.
Cellepora, 812.
Celloidin, 297 *et seq.*; the dry method, 303; clearing sections, 305; injection masses, 513, 514.
Cells, paper, for mounting, 432.
Cements, 430 *et seq.*; generalities, 430; comparative tenacity of, 433; APÁTHY'S, 455; BELL'S, 434; colophonium, 445; French, 442; HARTING'S gutta-percha, 441; KITTON'S, 436, 453; Knotting, 443; LOVETT'S, 454; MARSH'S gelatin, 431; MILLER'S, 435; STIEDA'S, 451; Tolu balsam, 450; turpentine, 444; ZIEGLER'S, 452.
Central nervous system, introduction, 728; hardening, 90, 729—742, 780, 880; imbedding and cutting, 743; general stains, 745—764; special stains, 765—776; mounting, 777, 778; the half-clearing method, 779; dry processes for preserving brains, 780; dissociation methods, 781, 782; neuroglia, 783; myelon of reptiles, &c., 784; and *see* Nerves, Retina, &c.; and for list of Authors reported *see* Neurological methods.
Cephalopoda, eyes, 816; ova, 613.
Cercariæ, 833.
CERFONTAINE, curarising *Lumbricus*, 841.
CERTES, Protozoa, 130, 858, 859.
Cestodes, 832.
Cestus, 855.
Chætopoda, 841.
Chætopteridæ, 841.
Chain section cutting, 281.
CHEATLE, dehydration apparatus, 2.
Cherry gum, 858.
CHICHKOFF, Turbellaria, 834.
CHIEVITZ, retina, 682.
China blue, 720.
Chinolin, blue, 126; hydrochlorate, 377.
Chironomus, salivary gland, 47; embryology, 620.
Chitin, clearing and softening, 823 *et seq.*
Chitonidæ, 817.
Chloral hydrate for narcotising, 14; preservative solutions, 365, 376, 394, 873; for preserving injection masses, 458; for maceration, 528, 685; for bleaching bichromate objects, 75; Gilson's mounting medium, 873; Geoffroy's ditto, *ibid.*
Chlorate of potash for maceration, 545.
Chloride-of-aluminium carmine, 145, 152.
Chloride of calcium mounting medium, 375.
Chloride of copper fluid, 64, 386.
Chloride of gold, *see* Gold chloride.
Chloride of magnesium for narcotisation, 17.
Chloride of palladium, 52, 84, 559, 746, 767.
Chloride of platinum, 23, 42, 50, 78, 83.
Chloride of vanadium, 683, 726.
Chloride of zinc, for hardening brain, 780.
Chlorine bleaching methods, 575—577.
Chloroform, for paraffin imbedding 275, 276; for celloidin ditto, 301; for bleaching, 582; for narcotisation, 11.

The numbers refer to the Paragraphs, not to the Pages.

CHOLODKOWSKY, embryology of Blattida, 619.
Chondrosia, 217.
Chromate of ammonia, 43, 81.
Chromate of lead stain, 234.
Chromate of silver impregnation, 704 *et seq.*
Chromates as fixing agents, 43.
Chromatin, reactions of, 53, 97, 109, 636, 638; male and female, 645.
Chromic acid, generalities, 30; fixing, 30; hardening, 69, 732; killing, 18; maceration, 534; decalcification, 559, 560, 570, 571; chromic acid with acetic acid, 31; with alcohol, 33, 70; with nitric acid, 39, 40, 571; with platinum chloride, 42; with osmic acid, 34, 71; with acetic and osmic acid, 35, 36, 71.
Chromic objects, action of light on, 30; bleaching, 30.
Chromo-acetic acid, 31; chromo-aceto-osmic, 23, 35, 36, 637; chromo-formic, 32; chromo-nitric, 39, 40; chromo-osmic, 34, 71; chromo-picric, 41, 72; chromo-platinic, 42, 83.
CHRSCHTSCHONOWIC, gold method, 228.
Chrysaurein, 95, 107.
CHUN, imbedding Siphonophora, 282.
CIACCIO, gold process, 228; motor plates, 670; corpuscles of Golgi, 674.
CIĄGLIŃSKI, spinal cord, 733, 743.
Cilia of Infusoria, 860, 861.
Ciliated epithelium, 821.
Ciona, 811.
Cladocora, 849.
Clavellina, 811.
Cleaning slides and covers, 864.
Clearing, generalities, 2, 99.
Clearing agents, 99, 342 *et seq.*; STIEDA'S experiments on, 343; NEELSEN and SCHIEFFERDECKER'S, 343—349; celloidin sections, 305; paraffin ditto, 283.
Clove oil, 4, 99, 345.
COBB'S differentiator, 2.
Cocain for narcotising, 15.
Coccidæ, 823.
Cochineal, theory of staining with, 144—146; use of, 147; HERRICK'S 154; CZOKOR'S, 154; KLEIN'S, 154; MAYER'S, 172, 173; PARTSCH'S 154.
Cochlea, 559, 562, 686, 687.
Cœlenterata, 849—856.
COHNHEIM, gold staining, 223; silver staining, 668.
COLE, freezing method, 317; gum medium, 399.
COLLIN, *Criodrilus*, 841.
COLLINGE, pelagic fish ova, 610.
Collodion imbedding, 297 *et seq*; section-fixing process, 325, 326, 340.
Collodionising paraffin sections, 282.
Colophonium, cement, 445; imbedding mass, 312; mounting medium, 422.
Comatula, 848.
Combination stains, 235 *et seq*; and *see* Stains, combination.
Congelation imbedding methods, 315, 322.
Congo red, 93, 95, 98, 132, 723—725.
Conjunctiva, 653.
Connective tissue, 138, 786 *et seq.*
CONTEJEAN, Ranvier's hæmatoxylin, 187.
Convoluta, 834.
COOK, hæmatoxylin, 192.
Copal, imbedding process, 311; varnish for mounting, 424.
Copepoda, 822.
Copilia, 822.
Copper, chloride and acetate fixing mixture, 64.
Copper sulphate fluids, fixing, 43, 44, 64; preserving and hardening, 64, 82; impregnation process, 234.
Coral, 311, 849.
Corallin, 95, 107.
CORI, narcotisation, 13, 15; Flemming's mixture, 35; preserving osmic acid solutions, 26.
Cornea, 121, 228, 653, 658, 659.
Corpuscles, tactile, 651 *et seq*; corpuscles of Golgi, 671—674; of Herbst and Grandry, 652; of Krause, 653.

The numbers refer to the Paragraphs, not to the Pages.

Corrosion, 555 *et seq.*; Altmann's methods, 558.
Corrosive sublimate, fixing liquids, 36, 47, 48; Lang's liquids, 48; preservative liquids, 379—385.
Covers and slides, cleaning, 864.
Cox, Golgi's mercuric impregnation, 761.
Creosote, *see* Kreasote.
Creseis, 813.
Criodrilus, 841.
Cristatella, 14.
Crocein, 95, 139.
Crystal violet, 98, 795.
Crystalline, 660.
Ctenophora, 45, 307, 855.
Cuccati, picro-carmine, 162; hæmatoxylin, 190; retina, 682, 683; soda-carmine, 167.
Cucumaria, 845.
Cupric fixing mixtures, 43, 44, 64, 854; hardening ditto, 82; preservative ditto, 64, 386; impregnation process, 234, 764.
Curare for narcotisation, 17, 633, 841.
Curschmann, amyloid matter, 109.
Cyanide of mercury, for fixing, 49.
Cyanin, 93, 95, 98, 126.
Cybulsky, gold method, 229; muzzle of ox, 650.
Cytological methods, 632 *et seq.*; methods of study, 632; subjects for ditto, 633; observation of living cells, 633, 634; staining ditto, 634; fresh cells, 635; microchemical reactions, 636; fixing, 64, 637; cytological stains, 638, 639; mounting, 642; Nebenkern, Archoplasmakugel, sphère attractive, 197, 208; nucleus of Balbiani, 640; cytology of the ovum, 644; spermatology, 645; methods of Altmann, 637, 641; Babes, 638, 643; Baumgarten, 643; Benda, 639; Beneden, van, 644; Beneden, van, and Neyt, 637, 639, 644; Bizzozero, 643; Boveri, 644; Carnoy, 635—637, 644, 645; Flemming, 633, 635, 637, 639, 645; Gehuchten, van, 637, 644; Gilson, 645; Grenacher, 642; Heidenhain, M., 633, 637, 639; Henneguy, 106; Hermann, 637; the Hertwigs, 644; Kultschitzky, 644; Nissen, 643; Peremeschko, 633; Pfitzner, 643; Platner, 639, 643; Rabl, 637, 639, 642; Schottländer, 643; Strasburger, 643; Tizzoni, 643; Uskoff, 643; v. la Valette St. George, 634, 644; Zacharias, 644; Zwaardemaker, 643.
Czerny, macerating mixture, 531.
Czokor, cochineal, 154; turpentine cement, 444.

D.

Dahlia, 93, 95, 98, 103, 789.
Dammar, 420, 421.
Davidoff, ova of *Distaplia*, 611.
Davies, injection, 471.
Daszkiewics, Korybutt, myelon of Reptilia, &c., 784; plasma-cells, 788.
Deane, mounting medium, 393; glycerin jelly, 406.
Decalcification, 559 *et seq.*
Decapoda, 822.
Decker, section stretcher, 280.
Deecke, hardening brain, 738; imbedding and cutting, 743.
Definition, affected by transparency of stains, 100; green light for, 866.
Degenerate nerves, 720 a.
Dehydration, 2, 3.
Dekhuysen, fat, 787; blood, 804; silver staining, 213.
Delafield, hæmatoxylin, 185.
Delage, osmium-carmine, 167; sulphate of iron for fixing, 834; Rhabdocœla, 834.
Deltapurpurin, 95, 133.
Dendy, *Geonemertes*, 835.
Dendrocœla, 834.
Dendrophyllia, 849.
Dennissenko, retina, 682.
Depigmentation, 575 *et seq.*; 823 *et seq.*

The numbers refer to the Paragraphs, not to the Pages.

Desilicification, 574.
DE SOUZA, pyridin, 90.
Dextrin freezing mass, 318.
DEZSÖ, *Tethya*, 856.
Differentiators, 2.
Diffusion apparatus, 2.
Diffusion currents, to avoid, 2.
Digestion, 549 *et seq.*
DIMMOCK, carminic acid, 144, 167.
Dinitrosoresorcin, 720.
DIOMIDOFF, hardening brain, 732.
Diphyes, 854.
DIPPEL, hæmatoxylin, 184.
Diptera, embryology, 620.
Dissections, minute, 4.
Dissociation, 522 *et seq*; of nervous tissue, 781.
Distaplia, 611.
DOGIEL, olfactive organs, 655; iris, 678; methylen-blue impregnation method, 115, 117, 119, 120, 652, 653; corpuscles of Herbst and Grandry, 652; retina, 683.
DOMERGUE, FABRE, mounting media, 401, 402.
DOSTOIEWSKY, iris, 679.
Double imbedding in celloidin and paraffin, 306.
Double stains, *see* Stains, Combination.
DOYÈRE, nerve and muscle of Arctiscoida, 826.
DRASCH, gold staining, 222; tactile hairs, 647.
DRASH, serial section method, 325.
DROOST, Mollusca, 821.
DUNHAM, clearing celloidin sections, 305.
DU PLESSIS, fixing method, 46; Nemertians, 835; Protozoa, 859.
DURHAM, alcohol section-fixing process, 324.
DUTILLEUL, picro-borax-carmine, 171.
DUVAL, silver staining, 213, 215; carmine and anilin blue, 243; collodion imbedding, 297 *et seq.*; embryology of Aves, 592; hardening encephalon, 734; purpurin, 201, 776; paraffin sections, flattening, 280; staining nerve-tissue, 749.

E.

Ear, inner, 34, 686, 687.
Eau de Javelle, 556, 581, 598, 616.
Eau de Labarraque, 557, 581, 598, 616.
EBNER, VON, decalcification, 563.
Echinodermata, 19, 635, 845, 848.
Echinorhyncus, 837.
Echtgelb, 95, 139.
Echtgrün, 720.
Echtroth, 95, 107.
EDINGER, nerve-centres, 77.
Egg-emulsion imbedding masses, 314, 321.
EHLERS, fixing fluid for Annelids, etc., 31, 841.
EHRENBAUM, imbedding method, 312.
EHRLICH, dahlia, 103; hæmatoxylin, 188; methylen blue for nerves, 114; plasma-cells, 788, 789, 804; gentian violet, 101; blood, 802, 804; classification of anilin dyes, 788.
EHRLICH-BIONDI-HEIDENHAIN stain, 259, 639, 759.
EHRMANN, structure of epithelium, 646.
EICHLER, labyrinth, 687.
EIJKMAN, degenerate nerves, 720a.
EISIG, fixing mixture, 83; narcotising, 13, 835.
EISMOND, quieting Infusoria, 858.
Elastic tissue, 104, 795.
Eledone, eyes, 816.
ELIEL, gum for labels, 865.
Embryology, general methods, 584—587; of *Agelena*, 622; *Amaræcium*, 612; Amphibia, 82, 590—603; Amphipoda, 626; Aphides, 621; Araneina, 622; Arthropoda, 616 *et seq.*; *Ascaris*, 54, 644; *Astacus*, 625; Aves, 589—594; *Axolotl*, 599; Blattida, 619; Cephalopoda, 613; *Distaplia*, 611; Diptera, 620; Echinodermata, 635, 848; Gastropoda, 614, 615; *Lacerta*, 39, 596; Lepidoptera, 618; *Limax*, 615; *Lumbricus*, 630; Mammalia, 588; Mollusca, 613 *et seq.*; *Orchestia*, 626; Phalangida, 623,

The numbers refer to the Paragraphs, not to the Pages.

624; Pisces, 604 *et seq.*; *Planaria*, 629; Porifera, 856; rabbit, 588; *Rana*, 603; Reptilia, 595—597; *Salamandra*, 602; *Tænia*, 628; Teleostea, 40, 584, 604—610; *Triton*, 600, 601; Tunicata, 611, 612; Vermes, 628 *et seq.*
Embryos, reconstruction from sections, 587.
Emery, injection, 510.
Encephalon, 728 *et seq.*; and *see* central Nervous System, Neurological Methods, etc.
Endosmosis, to avoid, 4.
Engelmann, ciliated epithelium, 821; green light in microscopy, 866.
Entire objects, preparation of, 4.
Eosin, 95, 98, 135; eosin and anilin green, 265; — and dahlia, 258, 266; — and gentian, 258; — and hæmatoxylin, 189, 251, 252; — and iodine green, 270; — and methyl green, 260, 261a; — and methyl violet, 267; — and silver nitrate, 270; eosin stains for blood, 804.
Epidermis of Mollusca, 814, 821.
Epithelium, 120, 121, 646 *et seq.*; ciliated, 821; glandular, 646.
Erlicki, hardening fluid, 77, 731.
Erythrosin, 135.
Eserin, for preserving Infusoria, 859.
Essence, *see* Oil, and Clearing agents.
Eternod, histological rings, 216.
Ether, for Preserving, 2.
Etherisation, 12.
Eulenstein, cement, 438.
Eunice, 841,
Examination media, 356 *et seq.*, 767.
Exner, hardening brain tissue, 732, 740.
Exosmosis, to avoid, 4.
Eyclesheimer, orientation of celloidin objects, 300; dry method of cutting same, 303; clearing, 305.
Eyes, of Arthropoda, 579, 583, 825; of Cephalopoda and Heteropoda, 816; of Chitonidæ, 817; of Gastropoda, 815; of *Pecten*, 818; of Vertebrates, 682 *et seq.*

F.

Fabre-Domergue, glucose preservative medium, 401; infusoria, 858, 859.
Fajerstajn, nerve-endings in tongue of frog, 657.
Faris, glycero-gum, 397.
Farrant's medium, 395, 396, 398.
Fat, 36, 787.
Faussek, critique of Flemming's mixture, 35.
Fecundation, artificial, 584.
Feist, methylen blue method, 119; orientating spinal cord, 743.
Ferria, elastic tissue, 795.
Ferrier, examination liquid for blood, 804.
Ferreri, phloroglucin decalcification, 562.
Fick, Golgi's silver chromate method, 712.
Fiedler, *Spongilla*, 856.
Fischer, soap imbedding mass, 833; Trematodes, 833; tactile corpuscles, 651; motor plates, 667.
Fixing, generalities, 2, 6 *et seq.*, 21 *et seq.*
Fixing agents, 25 *et seq.*; action of, 22; choice of, 23; methods of using, 24; washing out, 22, 24; acetic acid, 53; alcohol, 63; Altmann's methods, 37; alum, 45; bichloride of mercury, 47, 48; bichromate, 43; Carnoy's, 36, 54; chromates, 43; chromic mixtures, 31—36, 39—42; Cori's, 35; cyanide of mercury, 49; cytological fixing agents, 637; Du Plessis's method, 46; Flemming's, *see* Flemming; Max Flesch's, 34, 35; Fol's, 41, 52; Gilson's, 871; gold chloride, 52; heat, 7, 24; Hermann's, 51; iodine, 66; Kleinenberg's method, 57; Kultschitzky's, 43; Lang's, 48; Mayer's, 58, 59, 63; Merkel's fluid, 42; nitric acid, 37; nitric and chromic acid, 39, 40; nitrate of silver, 38; osmic acid, 25—29; Perenyi's liquid, 39;

The numbers refer to the Paragraphs, not to the Pages.

picric acid fluids, 41, 56—62; platino-acet-osmic mixture, 51; RIPART and PETIT'S solution, 64.
FLAGELLA, stains for, 861.
FLECHSIG, gold method, 228, 768; redwood nerve stain, 694; other other nerve stains, 763.
FLEISCHMANN, *Limax*, 813.
FLEMMING, chromo-acetic acid, 31; chromo-aceto-osmic acid, 23; weak solution, 35; strong ditto, 36; cytological methods, 633, 635, 637, 644, 645; damar solution, 420; double stains, 98, 236, 258; eyes of Gastropods, 815; fixing liquids, 31, 35—36a, 51, 53, 56, 61; imbedding mass, 291; preservation in glycerin mixtures, 2; injection of Molluscs, 820; staining method, 96—100, 101—103, 105, 107, 258; spermatological methods, 645; fatty tissue, 36, 787; bone, 800; mucous cells, 807; goblet cells, 809; connective tissue fibrils, 786.
FLESCH, MAX, chromo-osmic mixture, 34, 71; dichroism of hæmatoxylin stains, 178; double stain, 245; inner ear, 687; modification of Weigert's hæmatoxylin stain, 688, 689; staining nervous centres, 749; dry preservative process, 780; dental tissue, 801; blood, 803.
FLÖGEL, serial sections, 329.
FLORMAN, celloidin imbedding, 301.
Flustra, 812.
FOÀ, hæmatoxylin and safranin stain, 256; blood, 804.
FOETTINGER, chloral hydrate narcotisation method, 14; prepared paraffin, 286.
FOL, albumen fixative, 328; bleaching osmic objects, 29; chromo-aceto-osmic acid, 35; cleaning slides, 864; decalcification, 571; gelatin fixative, 334; glycerin jelly, 412; gold-impregnation of marine animals, 230; gumming labels, 865; imbedding *in vacuo*, 278; injection masses, 473, 480, 485, 493, 497; Infusoria, 52, 231, 859; narcotisation method, 19; paraffin oven, 277; perchloride of iron fixing and staining process, 52, 231; picro-carmine, 162; picro-chromic acid, 41, 57; reconstructing sections, 587; ribesin, 209.
Formic acid for fixing, 55.
FOSTER and BALFOUR, embryology of Aves, 589, 591.
Fowl, embryology of, 589—594.
FRANCOTTE, alcoholic carmine, 171; section-stretcher, 280; vacuum imbedding, 278; vegetable wax ditto, 287.
Frankincense, mounting medium, 429.
FREEBORN, staining nervous centres, 748; dissociation of ditto, 781; connective tissue, 786.
Freezing microtome, masses for, 315—322.
French cement, 442.
FRENZEL, serial section methods, 330, 336.
FREUD, gold process for nervous centres, 768.
FREY, ammonia-carmine, 165; injection mass, 491; iodised serum, 361; Parma blue, 123; indifferent liquids, 358.
FRIEDLAENDER, fixing mixture, 44.
Frog, bladder, 677; tongue, 657.
Fuchsin, 95, 107; ZIEHL'S carbolic, 107.
Fuchsin, S., 95, 98, 131; and *see* Säurefuchsin.

G.

GAGE, picro-carmine, 162; section-stretcher, 280; preservative fluid, 385; injection, 518; smooth muscle, 676; section fixing, 325; clearing mixture, 352; picric alcohol, 62, 540; preservation of potash or soda preparations, 529.
GALLEMAERTS, section-fixing process, 325.
GALLI, stain for neuroceratin funnels, 720.

The numbers refer to the Paragraphs, not to the Pages.

GANNAL, mounting medium, 373.
GARBINI, safranin staining, 101; double stain, 270; Alcyonaria, 849, cleaning slides, 864.
GASKELL, paraffin sections, water method, 280, 324.
Gastropoda, embryology, 614, 615; eyes, 815; preparation, 18, 813 *et seq.*; killing, 19, 814.
GAULE, section-fixing process, 324; hardening brain, 732; fixing liquid, 48; choice of paraffin, 285.
GEDOELST, digestion, 554a; medullated nerve, 720.
GEHUCHTEN, VAN, cytological methods, 637, 644; nerve centres, 717.
GEIGY & Co., chemical products, 179.
Gelatin, imbedding masses, 292—296, 319, 320; injection masses, 457 *et seq.*; cement, 431; section fixatives, 333, 334.
GELPKE, Weigert's hæmatoxylin, 688.
General method described, 2.
Gentian violet, 93, 95, 98, 102, 258.
GEOFFROY, mounting medium, 873.
Geonemertes, 835.
Gephyrea, 838.
GERLACH, gold method, 228, 767; gelatin imbedding, 295; injection, 468; embryology of Aves, 590; nerve-endings in muscle, 666.
GIACOMINI, gelatin process for serial sections, 778; dry process for preserving brains, 779.
GIBBES, HENEAGE, borax-carmine, 167; combination stains, 270; cleaning slides, 864.
GIERKE, ammonia-carmine, 165, 745; maceration of nerve-tissue, 532; staining nervous centres, 745, 757, 767; action of chloral on ditto, 75; neuroglia, 783; uranium carmine, 167; impregnation, 234.
GIESBRECHT, serial section method, 327; section-stretcher, 280; paraffin imbedding, 276.
GIESON, VAN, clearing celloidin sections, 305.
GILSON, acetate of uranium fixing liquid, 65; bleaching bichromate objects, 874; carmine blue, 123a; celloidin imbedding, 301, 303; chloral hydrate jelly, 873; cytological methods, 645; double imbedding, 306; Ehrlich-Biondi stain, 259; iodised osmic acid, 645; mercuro-nitric fixing mixture, 871; preservative fluid, 384; picro-alum carmine, 157, 872.
Glandular structures, 807 *et seq.*
Glucose preservative media, 401, 402.
Glue, marine, 440.
Glycerin, 403—413; glycerin mounts, to close, 430—432; glycerin and gum, 395—397; glycerin jelly, 406—413; preservative mixture, 2, 405.
Glycero-gum, 397.
GOADBY, preservative fluids, 382.
Goblet cells, 809.
GOETTE, hardening liquid, 82.
Gold chloride, for fixing, 52; for impregnation, generalities, 220—222, 229; composition of the commercial salt, 221; methods of BASTIAN, 228; BECKWITH, 768; BOCCARDI, 228, 670; BÖHM, 228; CARRIÈRE, 652; CHRSCHTSHONOWIC, 228; CIACCIO, 228, 670, 674; COHNHEIM, 223; CYBULSKY, 229; DRASCH, 222; FLECHSIG, 228; FREUD, 768; GERLACH, 228, 767; GOLGI, 670; HÉNOCQUE, 228; HOYER, 228; IGACUSCHI, 810; KOLOSSOW, 228; LÖWIT, 224; MANFREDI, 228, MAYS, 670; NESTEROFFSKY, 228; RANVIER, 225, 226; REDDING, 229; SCHIEFFERDECKER, 767; UPSON, 770, 771; VIALLANES, 227; ZIEHEN, 769.
Gold orange, 95, 139, 793.
Gold size, 439.
GOLGI, chromate of silver, Slow method, 704, 705; chromate of silver, Rapid method, 706; critique of the same, 707 *et seq.*; variations, —CAJAL, RAMÓN Y, 714, 715, 717; FICK, 712; VAN GEHUCHTEN, 717; GREPPIN, 710; HUBER,

The numbers refer to the Paragraphs, not to the Pages.

712, 877; KALLIUS, 877; KUPFFER, 810; LENHOSSÉK, 843; MONDINO, 713; NEUMANN, 710; OBREGIA, 711; OPPEL, 810; OYARZUN, 716; PETRONE, 713; SALA, 718; SAMASSA, 709; SEHRWALD, 708, 709, 711; corpuscles of Golgi, 671—674; mercuric impregnation of nervous centres, 760.

GOODALL, spinal cord, 880.

Gorgonia, 849.

GORONOWITSCH, embryology of Teleostea, 609.

GRAFF, VON, Turbellaria, 834.

GRAM, stain for bacteria, 101, 102.

Granule cells, 788 *et seq.*

Granules, protoplasmic, 93, 641, 788 *et seq.*

Grape sugar imbedding mass, 308.

GRASER, staining process, 111.

GRAVIS, section fixative, 332.

GRAY, serial sections, 333.

Green light in microscopy, 866.

GRENACHER, alum-carmine, 153; alcoholic carmine, 170; bleaching mixture, 583; borax-carmine, aqueous, 167; alcoholic, 169; castor oil for mounting, 425; hæmatoxylin, 185; purpurin, 201; eyes of Mollusca, &c., 816.

GREPPIN, Golgi's silver chromate method, 710.

GRIEB, alum-carmine, 153.

GRIESBACH, benzo-purpurin, 133; Bengal rose, 136; Biebricher Scharlach, 134; blood stains, 802—804; Congo red, 132; crocein, 139; double stain, 270; elastic tissue, 795; iodine green, 140; metanil yellow, 138; methyl green, 109; gold orange, 139; rocellin, 107; Säuregelb, 139; tropæolin, 107, 139.

GRÜBLER, histological reagents, 94, 863; cherry gum, 858; Ehrlich-Biondi-Heidenhain mixture, 639.

Grünpulver, 109.

GUIGNET, soluble Prussian blue, 477.

GULLAND, flattening and mounting paraffin sections, 280, 324; syrup and dextrin process, 868.

Gum arabic pure, 329; preservative media, 392—400; imbedding masses, 307—309, 315—317, 320; injection mass, 499; mucilage for labels, 865; fixative for sections, 329, 330.

Gum damar, 420, 421.

Gutta-percha, cement, 441; section-fixing process, 336.

H.

Hæmacalcium, 182.

Hæmalum, 180, 181.

Hæmammon, 183.

Hæmateïn, generalities, 174—179; properties, 179; produced by "ripening," 174—176; artifically prepared, 179; where obtainable, 179; formulæ for hæmateïn stains, 180, *et seq.* (alumina-hæmateïn stains, 180—192, other hæmateïn stains, 193—199); MAYER'S hæmalum, 180, 181; his hæmacalcium, 182; his hæmammon, or nitrate of ammonia hæmateïn, 183; and *see* "Hæmatoxylin."

Hæmateïn-ammoniak, 179.

Hæmateïn crystallisatum, 179.

Hæmatoxylic eosin, 189.

Hæmatoxylin, generalities, 174—179; theory of staining with, 145, 174—176, 178, 179; ripening of solutions, 174—176; dichroïsm of the stains, 178; affinities for plasma-granules and for mucin, 178; the practice of staining, 177; how to "blue" stains, 178; formulæ for stains,—alum solutions, 180—192; stains depending on the formation of other lakes, 193—199, and 688—703, 726, 755; APÁTHY'S, 194; ARNOLD'S, 192; BENDA'S, 690; BERLINER-BLAU'S, 688; BÖHMER'S, 186; BRANCA'S, 694; BREGLIA'S, 688, 694; BÜTSCHLI'S, 185, 196; COOK'S, 192; CUCCATI'S, 190; DELAFIELD'S,

The numbers refer to the Paragraphs, not to the Pages.

185; Dippel's, 184; Ehrlich's, 188; Flechsig's, 694; Grenacher's, 185; Haug's, 755; Heidenhain's, R., 193; Heidenhain's, M., 197; Hickson's, 192; Kaiser's, 876; Kleinenberg's, 184; Kultschitzky's, 697; Kupffer's, 810; Lissauer's, 696; Mallory's, 195; Mayer's, 180—183; Mercier's, 701; Minot's, 199; Mitchell's, 192; Pal's, 692; Prudden's, 185; Ranvier's, 187; Renaut's, 189; Rossi's, 698; Sanfelice's, 191; Schäfer's, 693; Squire's, 188a; Weigert's, 688—703; Wolter's, 699, 700, 726; and *see* the separate entries such as "Iron Hæmatoxylin," "Hæmacalcium," &c.; and *see* "Hæmateïn."

Haensel, liquid of, 41.

Hairs, tactile and others, 546, 646, 647.

Halicryptus, 838.

Haller, Béla, macerating fluid, 543.

Halliburton, chemistry of the cell, 636.

Hamann, carmine, 167; Echinoderms, 846; acanthocephali, 837.

Hamilton, freezing process, 316; Weigert's hæmatoxylin stain, 691; hardening brain, 737; freezing ditto, 743.

Hanaman, cleaning slides, 864.

Häntsch, glycerin medium, 405.

Hardening agents, 67 *et seq.*; acetate of lead, 87; alcohol, 88; bichromate of ammonia, 80; of potash, 75, 76; ditto with cupric sulphate, 77; chloride of palladium, 84; of platinum, 83; chromate of ammonia, 81; chromic acid fluids, 69—72; nitric acid, 74; osmic acid, 73, 732, 740; Erlicki's solution, 77; Merkel's solution, 83; Müller's solution, 76; generalities, 67 to 69; hardening nerve-centres, 729 *et seq.*; Perényi's solution, 39; picric acid, 86; pyridin, 90, 880.

Hardy, Rotifers, 839.

Harmer, silver staining marine animals, 217.

Hartig, white injection mass, 490.

Harting, preservative fluid, 379; cement, 441.

Haswell's Dehydration apparatus, 2.

Haug, lithium-carmine, 166; borax-carmine, 167; hæmatoxylin, 192, 755; decalcification, 559, 562, 568, 569; inner ear, 687; modification, of Weigert's nerve stain, 755.

Hayem, blood, 802, 803.

Heat, killing by, 7; fixing by, 24, 637.

Hegmons, embryology of Blattida, 619.

Heider, collodionising sections, 282.

Heidenhain, R., carmine, 165; hæmatoxylin, 193; anilin blue, 128; triple stain, 259; glands of small intestine, 811.

Heidenhain, M., cytological methods, 633, 637, 638, 639; lanthanin, 637; hæmatoxylin (iron), 197; discussion as to the Ehrlich-Biondi stain, 259; paraffin imbedding, 275; cause of "shrinkage" in paraffin sections, 280; water section fixing process, 324.

Helix, eyes, 815; ova, 614; sexual glands, 645; narcotisation, 16, 19.

Henchman, embryology of Limax, 615.

Henking, collodionsing sections, 282; embryology of Diptera, 620; of Phalangida, 624; of Insecta in general, 617; examination liquid for ova, &c., 617.

Henle, staining nervous tissue, 746.

Henneguy, alum-carmine, 156; embryology of Aves, 591; of *Axolotl*, 599; of Gastropoda, 614; of Mammalia, 588; of Teleostea, 604; sexual gland of *Helix*, 645; Protozoa, 857, 858; washing-out carmine stains, 149; permanganate staining method, 106, 149; mounting methyl green stains, 109; mounting paraffin sections, 280, 324, 328.

The numbers refer to the Paragraphs, not to the Pages.

Hénocque, gold process, 228.
Hermann, silver staining, 213; papillæ foliatæ of rabbit, 654; platino-aceto-osmic mixture, 23, 51, 637; osmium impregnation, 233; cytological stains, 639.
Hermann-Böttcher staining process, p. 60, note.
Herrick, cochineal alum-carmine, 154.
Hertwig, silver staining, 213, 217; macerating methods, 542; Actiniæ, 9, 849; Ctenophora, 307; Medusæ, 852, 853; embryology of *Rana*, 603; of *Triton*, 601; of *Strongylocentrotus*, 644; tobacco-smoke method, 9.
Herxheimer, elastic tissue, 795; epithelium, 646.
Heschl, amyloid matter, 109.
Heteropoda, eyes, 813, 816.
Heurck, van, mounting medium, 415.
Heydenreich, amber varnish, 447.
Heys, balsam, 418.
Hickson, hæmatoxylin, 192; maceration, 528; eye of *Musca*, 825.
Hirudinea, 16, 118, 844.
His, fixing, 37; cornea, 658.
Hochstetter, injection, 514.
Hofer, narcotisation method, 16.
Hoffmann, vacuum imbedding, 278.
"Hofmann's Grün," 140.
Hoggan, silver staining, 213; histological rings, 216; perchloride of iron stain, 231.
Holl, toluol for imbedding, 275; note concerning liquid of Kleinenberg, 637.
Holothurioidea, 18, 845.
Homarus, eyes, 825.
Hopewell Smith, decalcification, 565, 796, 797.
Hopkins, maceration, 540, 544.
Horn, 646.
Hoyer, ammonia-carmine, 165; neutral ditto, 165; picro-carmine, 162; silver staining, 213; gold ditto, 228; gum arabic mounting medium, 394; gelatin injection masses—carmine, 472; Berlin blue, 481; lead chromate, 484; silver nitrate, 486; green, 487; shellac mass, 516; oil-colour masses, 517; chloral for preserving masses, 458; water section-fixing process, 324; reactions of mucin, 807.
Huber, Golgi's silver chromate method, 712, 877.
Hudson, Rotifers, 839.
Huxley and Martin, ammonia-carmine, 165.
Hyatt, imbedding method, 310.
Hydatina, 839.
Hydra, 15, 16, 114, 849, 850.
Hydrate of chloral, for narcotising, 14; and *see* Chloral.
Hydrochlorate of Chinolin, 377.
Hydrochlorate of cocaïn, 15.
Hydrochloric acid for decalcification, 559, 563—566.
Hydrofluoric acid for desilicification, 574.
Hydrogen peroxide, for bleaching, 29, 30, 580.
Hydroidea, 7, 15, 16, 19, 850.
Hydroxylamin, 16.
Hypochlorite of potassium, *see* Eau de Javelle.
Hypochlorite of sodium, *see* Eau de Labarraque.

I.

Ide, double imbedding, 306.
Igacuschi, gold process for liver, 810.
Iijima, embryology of *Planaria*, 629.
Imbedding, defined, 2; generalities on, 2, 272 *et seq*; imbedding *in vacuo*, 278; double imbedding in celloidin and paraffin, 306; and *see* also Celloidin, Paraffin, &c.
Imbedding masses, albumen, 314, 321; aniseed, 322; bayberry tallow, 287; celloidin, 297 *et seq.*; collodion, *ibid.*; colophonium, 312; copal, 311; dextrin, 318; egg emulsion, 314; freezing masses, 315—322; gelatin, 292—296; grape sugar, 308; gum, 307, 309; gum and syrup, 316; paraffin,

The numbers refer to the Paragraphs, not to the Pages.

285—287; photoxylin, 297; shellac, 310; soap, 288, 291, 833; vegetable wax, 287; wax, 287.
Impregnation, distinguished from staining, 118, 210; negative and positive ditto, 210; primary and secondary ditto, 210.
Impregnation methods, 113—121, 210 *et seq.*; and *see* Gold chloride, Nitrate of silver, Methylen blue, &c.
Inchiostro di Leonardi, 111.
India-rubber cement, 442.
Indian ink injection, 512.
Indifferent liquids, 356 *et seq.*
Indigo, artificial, 125.
Indigo-carmine, 202; THIERSCH'S staining method, 203; SEILER'S, 241; MERKEL'S, 242.
Indirect staining method, 96 *et seq.*
Indulin, 95, 125.
Infusoria, 857 *et seq.* and *see* Protozoa.
Injection of leeches, 844.
Injection of Mollusca, 820.
Injections, 457 *et seq.*; injection masses, *albuminous*, JOSEPH'S, 498: *aqueous*, BJELOUSSOW'S gum, 499; EMERY'S carmine, 510; LETELLIER'S vanadate of ammonia, 511; MAYER'S blue, 509; MÜLLER'S blue, 508; RANVIER'S blue, 507; TAGUCHI'S Indian ink, 512: *collodion*, HOCHSTETTER'S, 514; SCHIEFFERDECKER'S, 513: *fatty*, HOYER'S oil colour, 517; TEICHMANN'S linseed oil, 519: *gelatinous*, BRÜCKE'S Berlin blue, 478; CARTER'S carmine, 470; DAVIES'S carmine, 471; FOL'S carmine, 473; FOL'S black (or brown), 493; FOL'S blue, 480; FREY'S white, 491; GERLACH'S carmine, 468; GUIGNET'S Prussian blue, 477; HARTIG'S white, 490; HOYER'S carmine, 472; HOYER'S Berlin blue, 481; HOYER'S chrome yellow, 484; HOYER'S silver yellow, 486; HOYER'S green, 487; MILLER'S purple, 494; RANVIER'S carmine, 466; RANVIER'S Prussian blue, 475, 476; RANVIER'S silver nitrate, 496; ROBIN'S vehicles, 458, 459; ROBIN'S anilin masses, 465; ROBIN'S cadmium, 463; ROBIN'S carmine, 460; ROBIN'S mahogany, 461; ROBIN'S Prussian blue, 462; ROBIN'S Scheele's green, 464; TEICHMANN'S white, 492; THIERSCH'S carmine, 469; THIERSCH'S Prussian blue, 479; THIERSCH'S lead chromate, 483; THIERSCH'S transparent green, 488; VILLE'S carmine, 467: *glycerin*, BEALE'S carmine, 501; BEALE'S Prussian blue, 503, 504; RANVIER'S Prussian blue, 505; ROBIN'S carmine, 502; ROBIN'S cadmium, 463; ROBIN'S mahogany, 461; ROBIN'S Prussian blue, 462; ROBIN'S Scheele's green, 464: *resinous*, BUDGE'S asphaltum, 515; HOYER'S shellac, 516; HOYER'S oil colour, 517: *starch*, PANSCH'S 518.
Injections, natural, 521.
Inner ear, 34, 559, 562, 686, 687.
Insecta, *see* Arthropoda.
Insecta, embryology, 617.
Intra-epidermic nerve-fibres, 649.
Intra-vitam staining, 93, 114, 115, 858.
Iodide of palladium nerve stain, 727.
Iodine, for fixing, 66; for hardening 89; LUGOL'S solution, 66; iodine as a mordant, 97.
Iodine green, 95, 98, 140.
Iodine hæmatoxylin, 190, 191.
Iodised osmic acid, 645.
Iodised serum, 360, 361; ditto for macerations, 523, 524.
Iris, 678, 679.
Iron, perchloride, for fixing, 52; for staining, 52, 231; pyrogallate of, 232; sulphate of, 44, 854; iron-hæmatoxylin stains, 196, 197.
ISRAËL, orceïn, 207.

J.

JACKSON, cloudiness in clearing media, 342.

The numbers refer to the Paragraphs, not to the Pages.

JACOBS, freezing mass, 320.
JACQUET, injection of leeches, 844.
JADASSOHN, structure of epithelium, 646; thionin for plasma-cells, 794.
JÄGER, glycerin medium, 405.
JAKIMOVITCH, silver staining, 214; medullated nerves, 720.
JAMES, cleaning slides, 864; damar solutions, 420.
JANSSENS, carmine blue, 123 a.
Japan wax, 287.
JAVELLE, eau de, *see* Eau de Javelle.
JELGERSMA, staining nervous centres, 757.
Jellies, glycerin, 406—413.
JENSEN, immobilisation of Infusoria, 858.
JENSON, spermatological methods, 645.
JOLIET, imbedding method, 307.
JOSEPH, injection mass, 498.
JOHNSON, LINDSAY, keeping osmic solutions, 25; sunning solutions of metallic salts, 25, 211; platinic hardening mixture, 78; gold impregnation, 228; Ehrlich-Biondi stain, 259, 687, 759; retina, 682, 683; mounting methyl green stains, 879; inner ear, 687; cementing celloidin blocks, 303.
Juice-canals, 120, 121.
JUNG, microtomes, 271.

K.

KADYL, imbedding mass, 290.
KAISER, glycerin jelly, 292, 294, 410; naphthylamin brown for spinal cord, 758; Bismarck-brown stain, 107; other nerve stains, 876.
KALLIUS. Golgi's silver chromate method, 877.
KANTHACK, histological reagents and apparatus, 94.
KASTSCHENKO, reconstruction from sections, 587.
KEISER, fixation of Acanthocephali, 49, 60.
KEMP, blood-plates, 805.
KENT, S., Infusoria, 66, 859.
KENT, A. F. S., making Farrant's medium, 395; cement, 456.
Kernschwarz, 208.
Killing tissues and organisms, 6—20; killing by heat, 7; by rapid fixing agents, 8; by narcotisation, 9; by nicotin, 10; by chloroform, 11, 12; by ether and alcohol, 12; by chloral, 14; by cocain, 15; by carbonic acid, 19; by asphyxiation, 19; by chromic acid, osmic acid, "Kleinenberg," or sublimate, 18; by fresh water, 20; by warm water, 20; by hydroxylamin, 16; by chloride of magnesium, 17; by curare and other drugs, 18.
KINGSLEY, imbedding method, 273.
KISHINOUYE, embryology of Araneina, 622.
KITTON, asphalt, 436; cement, 453.
KLEBS, glycerin jelly, 292, 293.
KLEIN, fixing mixture, 33; hardening intestine, 81; cornea, 658; cochineal, 154.
KLEINENBERG, colophonium for mounting, 422; embryology of *Lumbricus*, 630; picro-sulphuric acid, 57; hæmatoxylin, 184.
KLEMENSIEWICS, picro-carmine, 162.
KNAUER, cleaning slides, 864.
Knotting, for cement, 443.
KOCH, VON, copal imbedding method, 311.
KOGANEI, iris, 679.
KÖLLER, embryology of Aves, 593.
KÖLLIKER, ovum of rabbit, 588; bone, 800.
KOLLMANN, fixing mixture, 40.
KOLOSSOW, gold method, 228; osmium mixture, 28, 233.
KÖPPEN, elastic tissue, 795.
KOROTNEFF, killing Siphonophora, 11.
KORSCHELT, Infusoria, 859.
KORYBUTT-DASZKIEWICS, myelon of reptiles, 784; plasma-cells, 788.
KOSSEL, chemistry of the cell, 636.
KOSSINSKI, double stains, 269.
KOTLAREWSKY, central nervous system, 35, 87, 732.

The numbers refer to the Paragraphs, not to the Pages.

KOWALEWSKY, embryology of Teleostea, 607.
KRAUSE, corpuscles of, 653; molybdate of ammonium, 234; motor plates, 670; silver staining, 214; retina, 683, 685; spinal cord, 733; thiophen green, 141.
Kreasote, for bleaching, 578; for clearing, 352*a*; preservative fluids 368—370.
KROMAYER, methyl violet for epithelium, 111, 646.
KRYSINSKY, photoxylin imbedding, 297.
KRONECKER, artificial serum, 362.
KRÖNIG, cement, 445.
KÜHNE, digestion fluids, 554; macerating mixture, 545; motor plates, 670.
KÜHNE, H., aniseed freezing mass, 322; staining method, 98.
KUHNT, retina, 682, 683.
KÜKENTHAL, Annelids, 14, 840—842.
KULTSCHITZKY, fixing mixture, 43; double imbedding, 306; preservative fluids, 2; ovum of *Ascaris*, 644; tactile corpuscles, 651; stain for nervous centres, with hæmatoxylin, 697.
KUNSTLER, Protozoa, 859.
KUPFFER, embryological methods, 596; axis cylinder, 722; liver, 810.
KUSKOW, digestion fluid, 552.
KUTSCHIN, clearing method, 343.

L.

LABARRAQUE, eau de, *see* Eau de Labarraque.
Labelling slides, 865.
Labyrinth of ear, 559, 562, 686, 687.
Lacerta, ova of, 39.
Lactic acid for decalcification, 559, 569.
LAMANSKY, sensibility of the eye, 866.
Lamellibranchiata, aquiferous pores, 813; epithelium, 821; eyes, 818, 820; sexual gland, 645; shell, 311—313, 817, 819.
LANDOIS, impregnation methods, 234; macerating mixture, 532.
LANDOLT, retina, 685.
LANDSBERG, Infusoria, 859.
LANG, fixing liquids, 48; methods for Platyhelmia, 48, 834.
LANGERHANS, *Amphioxus*, 662; gum and glycerin medium, 396; tactile corpuscles, 651.
LANGHANS, dichroism of hæmatoxylin stains, 178.
LANKESTER, eyes of *Limulus*, 825.
LAVDOWSKY, bilberry juice stain, 209; myrtillus, 209; chloral hydrate medium, 376; macerating fluid, 528; inner ear, 687; methylen blue impregnation, 119.
LAWRENCE, glycerin jelly, 407.
Lead, acetate, 87, 732; chromate, 234.
LEBER, impregnation methods, 234.
LECLERCQ, spermatological stains, 645; blood stains, 804.
Leech, 844.
LEGAL, alum-carmine, 157.
LEGROS, silver staining, 215.
Lemon juice for fixing nuclei, 637.
LENHOSSÉK, VON, myelon of reptiles, 784; nerves of annelids, 843.
LENNOX, retina, 683.
Lens, crystalline, 660.
LÉON, nucina, 209.
LEONARDI, inchiostro di, 111.
Lepidoptera, embryology, 618.
LEPKOWSKY, gold method for teeth and bone, 798.
LETELLIER, injection, 511.
LEUCKHART, imbedding boxes, 273.
LEVEN, saffron stain, 205.
Levulose for mounting, 402.
LEWIS, BEVAN, *see* under Neurological methods.
Lichtblau, 123.
Lichtgrün, 109.
LICHTHEIM, staining with Weigert's hæmatoxylin, 688.
LIEBERMANN, analysis of carmine, 144; carminic acid, 145.
Light, action of, on alcohol containing chromic objects, 30; on metallic

The numbers refer to the Paragraphs, not to the Pages.

salts, 25, 211; green, in microscopy, 866.
LILIENFELD, blood-plates, 805.
LILIENFELD and MONTI, test for phosphorus in tissues, 636.
Limax, 814; embryology, 615.
Limulus, 825.
LINDSAY JOHNSON, *see* JOHNSON, LINDSAY.
Linseed oil injection, 519.
Liquid of Müller, of Erlicki, of Kultschizky, of Merkel, &c., *see* the names of the respective authors.
Liquidambar, 427.
LISSAUER, hæmatoxylin nerve stain, 696, 876.
LIST, hæmatoxylin and eosin, 251; ditto and nitrate of rosanilin, 255; other combination stains, 261—265; macerations, 525; Anthozoa, 819; Coccidæ, 823; mucus cells, 808; goblet cells, 809.
Lithium-carmine, 166.
Litmus stain, 209.
Liver, 810.
Lizzia, 851.
LO BIANCO, S., *see* BIANCO, S., LO.
LOCY, embryology of *Agelena*, 622.
LOEFFLER, stains for flagella, 861.
LÖNNBERG, *Triænophorus*, 832.
LOEWE, crystalline, 660.
LOEWENTHAL, nerve-centres, 77; picrocarmine, 162.
LOEWY, macerating skin, 646.
Loligo, eyes, 816.
LONGHI, eserin for Infusoria, 859.
LONGWORTH, corpuscles of Krause, 653.
LOOSS, softening chitin, 557; Nematodes, 836.
LOVETT, cement, 454.
LÖWIT, gold method, 224, 667; blood, 802—804.
Loxosoma, 217, 812.
LUGOL, solution of, 66.
Lumbricus, 118, 840—843; embryology, 630.
LUSTGARTEN, elastic tissue, 104, 795.
LUYS, noir Colin, 757.
Lymphatics, 120, 121; and *see* Injections.

M.

MAAS, carmine and malachite green, 244.
MACALLUM, *Sphyranura*, 833; double stain, 242.
MAC BRIDE, development of *Amphiura*, 848.
Maceration, 522—548, 662; nervous tissue, 781; epithelium, 646; mollusca, 821.
Macrotoma, 829.
Magdala red, 95, 98, 107.
Magenta, 95, 98, 107.
MAGINI, stain for nervous centres, 762.
Magnesium chloride, for narcotisation, 17.
MÄHRENTHAL, VON, osmium impregnation, 233.
Malachite green, 95, 98, 143, 639.
MALASSEZ, ammonia-carmine, 164.
MALFATTI, chemistry of the cell, 636.
MALLORY, phospho-molybdic acid hæmatoxylin, 195.
Mammalia, embryology, 588.
Manchester brown, 110.
MANFREDI, gold method, 228.
MARCHI, VON, *Limax*, 814; corpuscles of Golgi, 672; degenerate nerves, 720a.
Marine animals, killing, 20; fixing, 24, 57; silver impregnation, 217; gold ditto, 230.
Marine glue, 440.
MARK, collodionising sections, 282; embryology of *Limax*, 615; section-fixing, 327.
MARSH, bleaching, 576; gelatin cement, 431; decalcification mixture, 571.
MARSHALL, May's gold process, 670.
MARTIN, benzoazurin, 107, 127.
MARTINOTTI, safranin straining, 101; damar solutions, 420; anilin black, 757; picro-nigrosin, 757; elastic tissue, 795; intra vitam staining, 93.
MASON, nervous system of Reptiles, &c., 742.

The numbers refer to the Paragraphs, not to the Pages.

Mastzellen, 788—794.
Maturation of ovum, 644; and *see* Cytological methods.
MATSCHINSKY, bone, 796.
MAURICE and SCHULGIN, double stain, 243; embryology of *Amarœcium*, 612.
Mauvein, 95, 107.
MAX FLESCH, *see* FLESCH.
MAYER, PAUL, acidulated alcohol, 63; alcoholic carmine, 170; prep. of Arthropods, 5, 58, 63, 822; albumen fixative for sections, 328; bleaching, 575; Bismark brown, 110; carmine staining, theory of, 144—147, 174; Carmalum, 151; Chloride-of-aluminium carmine, 152; Paracarmine, 168; cochineal, old formula, 146, 172; new formula, 146, 173; desilicification, 574; general principles of preparation, 5, 24; hæmatoxylin (hæmateïn) staining, theory of, 145, 174—179; Hæmalum, 180, 181; Hæmacalcium, 182; Hæmammon or Nitrate-of-ammonia Hæmateïn, 183; preparation of the Ammonia-salt of Hæmateïn, 179; dichroïsm of hæmateïn stains, 178; blueing them, 178; critique of Kleinenberg's hæmatoxylin, 184; injection, 508; picro-carmine, 162; picro-sulphuric acid, 55; picro-hydrochloric acid, 59; picro-nitric acid, 58; section-stretcher, 280; section fixing methods, 327, 328; shellac fixative, 327; water-bath for paraffin, 277.
MAYER, S., "violet B." for connective tissue, 129, 786; methylen blue method, 117, 119, 121.
MAYS, nerve-endings (gold process), 670.
MAYSEL, Bismarck brown, 110.
Medullated nerve, 688—727; and *see* Neurological methods.
Medusæ, 11, 18, 45, 542, 851—853.
MERCIER, hæmatoxylin nerve stain, 701; Upson's nerve-impregnations, 770, 771.
MERCK, carminic acid, pure, 148; chemical products, 179.
Mercury, bichloride, 47, 48 *et seq.*; and *see* Corrosive sublimate; biniodide, mounting medium, 414; cyanide, for fixing, 49.
MERK, making up chromo-aceto-osmic acid, 36.
MERKEL, chromo-platinic liquid, 42, 71, 83; impregnation method, 234; carmine and indigo stain, 242, 749; celloidin imbedding, 297; half-clearing brain sections, 779; staining nervous tissue, 746.
Metagelatin, 497.
Metallic salts, action of light on solutions, 25, 211.
Metallic stains, 210 *et seq.*
Metanil yellow, 95, 138.
Methanilin green, 109; methanilin violet, 111.
Methyl alcohol, for narcotising, 13; for safranin staining, 101.
Methyl green, 95, 98, 109, 879; combination stains with, 259—263.
Methyl mixture for maceration, 548.
Methyl violet, 93, 95, 98, 107, 111, 258.
Methylal, for dehydration, 2, 119.
Methylated spirit, 2, 862.
Methylen blue, for hardened central nervous system, 751—753, 773, 774; for plasma cells, 792; for impregnation, 95, 113 *et seq.*, 878; generalities, 113; staining in toto during life, 114; staining nerve-tissue during life, 115—121; influence of oxygen and of ammonia, 115; staining by injection or by immersion, 116; the solutions employed, 117, 118; preservation of the stain, 119; impregnation of epithelia, lymph-spaces, &c., 120, 121; stains for hardened nervous tissue (REHM), 751—753; (SAHLI), 773; (ADAMKIEWICS), 774; methods of APÁTHY, 115, 117, 118, 119, 878; ARNSTEIN, 117, 119; BIEDERMANN, 117, 666; BÜRGER, 835; DOGIEL, 115, 117, 119, 120, 652,

The numbers refer to the Paragraphs, not to the Pages.

653; Ehrlich, 114; Fajerstajn, 657; Feist, 119; Gerlach, 666; Lavdowsky, 119; Mayer, S., 117, 119, 121; Parker, 119; Retzius, 117, 119; Zoja, 114.
Meyer, salicylic solutions, 391.
Mibelli, elastic tissue, 795.
Microchemistry of the cells, 636.
Microtomes, 271, 867.
Migula, glycerised serum, 363.
Miller, vegetable wax, 287; injection, 494.
Milnesium, 826.
Minot, hæmatoxylin stains, 199; clearing celloidin sections, 305; macerating skin, 646; carmine, 167; serial sections, 777.
Minot, microtome, 271.
Mitchell, hæmatoxylin, 192.
Mitrophanow, gold process, 646; maceration, *ibid.*; sense organs of Amphibia, 656; prickle-cells, 646.
Möbius, macerating media, 539.
Moerner, tracheal cartilage, 801.
Moleschott, potash or soda solution, 529; Moleschott and Piso Borme's macerating fluid, 527.
Molgula, 811.
Mollusca, 813—821; aquiferous pores, 813; embryology, 613 *et seq.*; eyes, 815—819; fixation, 813; injection, 820; maceration, 538, 543, 821; removing mucus from, 814; shell, 311—313, 817, 819.
Molluscoida, 7, 14, 15, 812; and *see* Bryozoa.
Molybdate of ammonium, 234, 637.
Monadina, 859.
Mondino, medullated nerves (Golgi's method), 713.
Monobromide of naphthalin, 415.
Monti, sulphate of copper impregnation method, 764; test for phosphorus in tissues, 636.
Moore, stain for blood, 804.
Mordants for anilin dyes, 97, 101, 106.
Morgan, ova of Arthropods, 616, 823; removing albumen frum ova, 603.
Morphia for narcotising, 18.
Moseley, shell, 819; Chitonidæ, 817.
Mosso, blood, 803.
Motor plates, 665 *et seq.*
Mounds of Doyère, 826.
Mounting media, *see* Examination media.
Mucilage for labels, 865.
Mucin, 178, 807 *et seq.*
Mucus, removing, from Mollusca, 814.
Mucus glands, 104, 105, 252, 807 *et seq.*
Mueller, blood, 302, 804.
Müller, hardening liquid, 76, 536; silver staining, 214; injection mass, 508.
Münder, G., his histological reagents, 94.
Munson, chloral hydrate fluid, 376.
Musca, eyes, 825.
Muscle, dissociation of, 544 *et seq.*, 662; nerve-endings in, 665 *et seq.*; smooth, 676 *et seq.*; sections, 661.
Myelin, 719.
Myelon, *see* Central nervous system.
Myrtillus, 209.
Myrtle wax, 287.

N.

Nails, 646.
Nansen, maceration of nerve-tissue, 532.
Naphtha, for clearing, 275, 283, 354, preservative fluids, 370, 371.
Naphthalin, monobromide, 415.
Naphthalin red, 95, 107.
Naphtholorange, 107.
Naphthylamin brown, 758.
Naples Zoological Station methods, 5, *sub. fin.*, 811.
Narcotisation, 9—19, 811, note, 870.
Nealey, bone, 796.
Nebenkern, 208, 634, 639, 645.
Negro, motor plates, 670.
Nematodes, 557, 836.
Nemertina, 7, 835.
Nerves, degenerate, 720a.
Nerves, medullated, 115 *et seq.*, 688—727; and *see* Neurological Methods.

The numbers refer to the Paragraphs, not to the Pages.

Nerve-endings in muscle, 665 *et seq.*; in skin and others, 648 *et seq.*, 657; methylen blue method, 115 *et seq.*; and *see* Neurological Methods.
Nervous system, central, *see* Central Nervous System and Neurological Methods.
Nessler, test for ammonia, 467, note.
Nesteroffsky, gold process, 228.
Neumann, Golgi's silver chromate method, 710.
Neuroceratin, 704, 706, 720.
Neuroglia, 783.
Neurological methods, Adamkiewics, 774; Apáthy, 115, 117, 118, 119, 785, 878; Angelucci, 682; Alt, 724; Armanni, 694; Arnstein, 117, 119; Barrett, 682; Bechtereff, 732; Beckwith, 768; Beevor, 691, 692; Bellonci, 741; Bernheimer, 683; Benczur, 756; Benda, 690; Berlinerblau, 688; Betz, 165, 739, 745; Biedermann, 117, 666, 667; Boccardi, 670; Boll, 767; Boveri, 720; Bramwell, 779; Branca, 694; Breglia, 688, 694; Bremer, 670; Burckhardt, 742; Cajal, Ramón y, 669, 683, 684, 714, 715, 717; Carrière, 652, 781; Cattaneo, 673; Chievitz, 682; Ciaccio, 228, 670, 674; Ciaglinski, 733, 743; Cox, 761; Cuccati, 683; Cybulsky, 650; Daszkiewics, 784; Deecke, 738, 743; Dennissenko, 682; Diomidoff, 732; Dogiel, 115, 117, 119, 120, 652, 653, 655, 683; Duval, 734, 749, 776; Ehrlich, 114; Eijkman, 720a; Exner, 732, 740; Fajerstajn, 657; Feist, 119, 743; Fick, 712; Fischer, 651, 667; Flechsig, 228, 694, 763, 768; Flesch, Max, 34, 71, 687—689, 749, 780; Freeborn, 748, 781; Freud, 768; Galli, 720; Gaule, 732; Gedoelst, 720; van Gehuchten, 717; Gelpke, 688; Gerlach, 228, 666, 767; Giacomini, 778, 780; Gierke, 75, 532, 745, 757, 767, 783; Golgi, (chromate of silver impregnation, Slow Method) 704, 705, (Rapid Method) 706, (critiques and variations) 707—718, 810, (corpuscles of) 671—674, (gold impregnation) 670, (sublimate impregnation) 760, 761; Goodall, 880; Greppin, 710; Hamilton, 316, 691, 737, 743; Haug, 687, 755; Henle, 746; Hermann, 654; Huber, 712, 877; Igacuschi, 810; Jakimovitch, 720; Jelgersma, 757; Johnson, Lindsay, 682, 683, 759; Kaiser, 757, 876; Kallius, 877; Korybutt-Daszkiewics, *see* Daszkiewics; Kotlarewsky, 35, 87, 732; Krause, 670, 683, 685, 733; Kühne, 670; Kuhnt, 682, 683; Kultschizky, 651, (hæmatoxylin stain) 697; Kupffer, 722; Lavdowsky, 119, 687; Lenhossék, 784, 843; Lennox, 683; Lewis, B., 728, (hardening) 731, 736, 740, 743, (staining) 757, (half clearing method) 779, (compression method) 782; Lichtheim, 688; Lissauer, 696; Loewenthal, 77; Longworth, 653; Löwit, 224, 667, 670; Luys, 757; Magini, 762; Mallory, 195; Manfredi, 228, 672; Marchi, 672, (degenerate nerve) 720a; Marshall, 670; Martinotti, 757; Mason, 742; Mayer, S., 117, 119, 121; Mays, 670; Mercier, 701, 770, 771; Merkel, 746, 749, 779; Mitrophanow, 646, 656; Mondino, 713; Monti, 764; Nansen, 532; Negro, 670; Nesteroffsky, 228; Neumann, 710; Nikiforow, 775; Nissl, 723; Obersteiner, 728, (hardening) 731, (staining) 745, 750; Obregia, 711; Ogata, 732; Osborn, 743, 778, 784; Oyarzun, 716; Pal, 692, 760; Paladino, 727; Paneth, 688;

The numbers refer to the Paragraphs, not to the Pages.

Petrone, 713; Platner, 52, 720; Politzer, 687; Prenant, 686; Pritchard, 687; Ramón y Cajal, *see* Cajal; Ranvier, (gold methods) 225, 226, 647, 649, 667, 668, 671, 682, 685, 720; Rehm, 725, 751—753; Retzius, 117, 119; Rezzonico, 704, 720; Rossi, 698; Rutherford, 735; Ruffini, 673; Sachs, 696, 670; Sahli, 730, 731, 773; Sala, 718; Samassa, 709; Sankey, 757; Schäfer, 693; Schaffer, 683; Schiefferdecker, 584, 682, 685, 720, 767, 781; Schmaus, 747, 757; Schmidt, 784; Schultze, Max, 685; Schwalbe, 686; Sehrwald, 708, 709, 711; de Souza, 90, 880; Squire, 724; Stilling, 781; Stirling, 757; Tafani, 687; Tal, 760; von Thanhoffer, 782; Thin, 685; Tizzoni, 720; Trinchese, 667; Trzebinski, 732; Tschisch, 77; Upson, 748, (gold and iron method) 770, (gold and vanadium method) 771; Vasale, 695; Vejas, 757; Waldeyer, 687; Weigert, (hæmatoxylin impregnation) 688—703, (the 1885 method) 688, (the 1891 method) 703, (variations) 689—702, 755, (mounting sections) 305, 340, 426, 703, (hardening) 730, (Säurefuchsin method) 772, (retina) 683; Wolff, 670, 677; Wolters, 699, 700, 726; Ziehen, 769; Zoja, 114; Zuppinger, 756; and *see* Impregnation Methods, Retina, Inner Ear, Nerves, Central Nervous System, &c.

Neutralisation of carmine masses, 467.

Neutrophilous cell-elements, 788.

Neyt, *see* Beneden, van.

Nias, cleaning slides, 864.

Nietzki, carminic acid, 145; hæmateïn, 174.

Nicotin for narcotisation, 10.

Nigranilin, 95, 130.

Nigrosin, 95, 98, 125.

Nikiforow, borax-carmine, 167; stain for nervous centres, 775; clearing celloidin sections, 305.

Nissen, gentian violet staining process, 102; cytological methods, 643.

Nissl, axis cylinder, 723.

Nitrate of ammonia hæmateïn stain, 183.

Nitrate of silver, for fixing, 38; for impregnation, generalities, 212; methods of Alferow, 213; Cohnheim, 668; Dekhuysen, 213; Duval, 213, 215; Harmer, 217; the Hertwigs, 213, 217; the Hoggans, 212, 213, 216; Hoyer, 213; Jakimovitch, 214; Krause, 214; Legros, 215; Müller, 214; Oppitz, 214; Ranvier, 212, 213; von Recklinghausen, 213; Reich, 213; Robinski, 213; Rouget, 213, 214; Sattler, 214; von Thanhoffer, 214; Tourneux, 213; Vosmaer, 217; and *see* Golgi.

Nitrate of uranium, fixing mixture, 28; use with osmium, 78.

Nitric acid for fixing, 37—40, 637; for hardening, 74, 780; for maceration, 544, 545; for decalcification, 560, 561; for bleaching, 579.

Nitric and chromic acid mixtures, 39, 40.

Nitrite of amyl as a vaso-dilatator for injections, 500.

Noir Colin, 95, 130, 757.

Noll, corrosion, 556; sponge spicules, 856; salicylic acid medium, 392.

Nordmann, plasma-cells, 790.

Normal salt solution, 359.

Norris and Shakespeare, carmine and indigo stain, 242.

Nucina, 209.

Nuclein, reactions of, 636, and *see* Chromatin.

Nucleus, *see* Cytological methods.

Nychtoterus, 858.

The numbers refer to the Paragraphs, not to the Pages.

O.

Obersteiner, central nervous system, 728; hardening, 731; staining, 745, 750.
Obregia, Golgi's silver chromate method, 711; serial section method, 868.
Oceania, 851.
Octopus, eyes, 816.
Odenius, tactile hairs, 546.
Ogata, hardening brain, 732.
Ohlmacher, reactions of safranin with iodine and picric acid (pseudo-carcinoma corpuscles), 101; section fixing process, 869.
Oil, of aniseed, imbedding mass, 322; of bergamot, 347; cannel, 346; cedar wood, 344; cloves, 4, 343, 345; origanum, 305; sandal-wood, 349; turpentine, 343; and *see* Clearing agents.
Olfactive organs of Vertebrates, 655.
Ommatostrephes, eyes, 816.
One-third alcohol, 63, 525, 862.
Onuphis, 841.
Opalina, 858.
Opheliadæ, 841.
Ophiuridea, 847.
Oppel, liver, 810.
Opisthobranchiata, 813.
Opisthotrema, 833.
Oppitz, silver staining, 214.
Optic nerve, 741.
Orange, 95, 98, 107, 258, 259.
Orceïn, 207, 795.
Orchella, 206.
Orchestia, embryology, 626.
Origanum oil, 305, 348.
Orseille, 206.
Orseillin, 95, 107.
Orth, methyl violet, 111; lithium-carmine, 166; picro-lithium carmine, 240.
Osborn, embryology of *Triton*, 600; brain of Urodela, 743, 784; serial sections, 778.
Osmic acid, for fixing, 21, 23, 25—29; for hardening, 73, 732, 740, 741; for macerating, 541, 542; for impregnation, 233; blackening of preparations with, 29, 36, 575; how to keep solutions, 25, 26, 28, 35, 36; Cori's solution, 26; Kolossow's solution, 28; osmic acid and alcohol, 28; and chromic acid mixture, 34, 73; and chromic and acetic acid mixture, 35, 36; and iodine mixture, 645; acetic acid mixture, 28, 542; osmic and oxalic stain, 233; osmic and pyrogallic ditto, 233, 639.
Osmium-blackened fat, 36, 787.
Osmium-carmine, 167.
Osmosis, to avoid, 4, 358.
Ostracoda, 822.
Ova, pelagic, 83, 610.
Overton, bleaching osmic objects, 29; fixing with iodine vapours, 66.
Oviatt, injection method, 500.
Ovum, maturation of, 644; and *see* Cytological methods.
Ovum of *Ascaris*, 54, 644; other ova, *see* Embryology.
Owen, preservative liquid, 383.
Oxalic acid for maceration, 547.
Oxygenated water, 29, 30, 580.
Oyarzun, Golgi's silver chromate impregnation, 716.

P.

Pacini, preservative liquids, 380.
Pagenstecher, fixing fluid, 45.
Pal, hæmatoxylin stain, 692; staining nervous centres, 760.
Paladino, iodide of palladium nerve stain, 727.
Palinurus, ova, 58.
Palladium chloride, 52, 84, 234, 559, 746, 767.
Palladium iodide nerve stain, 727.
Palythoa, 849.
Pancreatin digestion fluids, 553.
Paneth, making Weigert's hæmatoxylin, 688; goblet-cells, 809.
Pansch, injection-mass, 518.
Paper, imbedding trays, to make, 273; paper cells, 432.
Papillæ foliatæ, 654.

The numbers refer to the Paragraphs, not to the Pages.

Paracarmine, 145, 168.
Paraffin, imbedding processes, 272 *et seq.*; review of, 284; paraffin masses, 285—287; solvents of, 273, 283; ovens, 277.
Paris green, 109.
Paris violet, 111.
Parker, turpentine cement, 444.
Parker, G. H., dehydration methods, 2; methylen blue method, 119; eyes of scorpions, bleaching, 579; eyes of *Homarus*, 825.
Parma blue, 95, 123.
Partsch, cochineal stain, 154.
Patten, embryology of Blattida, 619; eyes of Molluscs and Arthropods, 818; maceration of Mollusca, 821.
Paulsen, goblet cells, 809.
Pecten, eyes of, 818.
Pedicellina, 217, 812.
Pelagic ova, 83, 610.
Pennatulidæ, 849.
Pentacrinus, 848.
Pepsin digestion fluids, 449—552.
Perchloride of iron, for fixing, 52; for impregnation, 52, 231.
Peremeschko, cytological methods, 633.
Perenyi, fixing fluids, 39.
Pergens, picro-carmine, 162.
Periplaneta, ova of, 616, 823.
Perl, soluble carmine, 167.
Permanganate of potash, 46, 97, 106, 214, 533, 658; for fixing, 46; for washing out carmine stains, 149; for mordanting, 106, 149; for reducing silver impregnations, 214.
Perophora, 811.
Peroxide of hydrogen, 29, 30, 580.
Perrier, narcotising *Lumbricus*, 841.
Perruthenic acid, 809.
Petit, *see* Ripart and Petit.
Petrone, cerebro-spinal nerves, 713.
Pfitzer, imbedding mass, 291.
Pfitzner, safranin solution, 101; gold chloride and safranin, 270; damar solution, 420; *Opalina*, 859; cytological methods, 153, 643.
Phalangida, 828; ova of, 282, 623, 624.
Phallusia, 811.
Phascolosoma, 838.
Phénicienne, la, 110.
Phenylen brown, 110.
Phillipson, macerating skin, 646.
Phloroglucin, 562, 687.
Phoronis, 838.
Phospho-molybdic-acid hæmatoxylin, 195.
Phosphoric acid for decalcification, 559, 568.
Phosphorus in tissues, test for, 636.
Photoxylin imbedding method, 297, 299.
Phyllosoma, 822.
Physalia, 854.
Physiological salt solution, 359.
Physophora, 854.
Pianese, double-stain for connective tissue, 786.
Picric acid, fixing fluids, 2, 56—62; hardening ditto, 86; staining with, 137, 236—240; decalcification with, 559, 567.
Picric alcohol, 62, 540.
Picro-alum-carmine, 157, 872.
Picro-carminate of ammonia, 159, 160.
Picro-carminate of soda, 162.
Picro-carmine, 159—162, 388.
Picro-chromic acid, 41, 72.
Picro-chromo-sulphuric acid, 57, 60.
Picro-hydrochloric acid, 59, 567.
Picro-lithium carmine, 240.
Picro-nigrosin, 757, 786.
Picro-nitric acid, 58, 567.
Picro-osmic acid, 61.
Picro-sulphuric acid, 57, 567.
Pictet, examination liquid, 645; spermatological methods, *ibid.*
Piersol, embryology of Mammalia, 588.
Pigment spots, artificial, 77, 731, 732.
Pintner, Tæniæ, 832.
Pisenti, alum-carmine, 153.
Plagiostomidæ, 834.
Plakina, larvæ, 856.
Planaria, 48, 629.
Plasma-cells, 178, 788—794.
Plastic reconstruction of sections, 587.

The numbers refer to the Paragraphs, not to the Pages.

Plastin, 636.
Platinic mixture, MERKEL'S, 83; LINDSAY JOHNSON'S, 78.
Platino-aceto-osmic mixture, 23, 51, 637.
Platino-osmo-bichromate mixture, 78.
Platinum chloride, 23, 42, 50, 78, 83, 85.
PLATNER, Kernschwarz, 208; Nebenkern, 639; medullated nerve, 52, 720.
Platyhelmia, 48, 832—835.
PLESSIS, DU, *see* DU PLESSIS.
Plutus, 155, 848.
Podocoryne, 851.
PODWYSSOZKI, fixing mixture, 36; safranin, 101; carcinoma corpuscles, 101.
Poisoning methods, 18.
POLAILLON, impregnation method, 231.
POLI, serial sections, 335; imbedding mass, 291.
POLITZER, inner ear, 687.
Polychæta, 841.
Polycladidea, 834.
PÖLZAM, imbedding mass, 289.
Ponceau, 98.
Porifera, 856.
Porpita, 854.
Potash, acetate, mounting medium, 374.
Potash solution for maceration, 529.
Potassium bichromate, 75 *et seq.*
Potassium hypochlorite, 556, 581, 598, 616.
Potassium permanganate, *see* Permanganate of potash.
POUCHET, bleaching methods, 578, 580.
PRENANT, safranin staining, 101; spermatological methods, 645, cochlea, 686.
Preservative media, 2, 356 *et seq.*
Priapulus, 838.
Prickle-cells, 646.
Primrose, 135.
PRINGLE, the water section-fixing process, 324; vacuum imbedding, 278; double stain, 254.
PRITCHARD, hardening fluid, 70; reducing solution, 228; inner ear, 687.
Prosobranchiata, 813.
Protopterus, 742.
Protula, 841.
Protozoa, introductory, 857; killing, 7, 859; quieting, 857; staining *intra vitam*, 858; cilia, 860; flagella, 861; other methods, 857—859.
PRUDDEN, hæmatoxylin, 185.
Prussian blue, soluble, 476, 477; impregnation with, 234; injection-masses, *see* Injections.
Prussic acid, 18.
Pteropoda, 813.
Purpurin, 201, 776.
Putrefied carmine, 165.
Pyrenin, 636.
Pyridin for hardening and clearing, 90, 880.
Pyrogallate of iron stain, 232.
Pyroligneous acid, for hardening, 682.
Pyrosin, 135.
Pyrosoma, 7, 307.

Q.

QUEKETT, preservative fluid, 371.
Quieting Infusoria, 857.
Quince-mucilage fixative, 331.
Quinolein, 93, 95, 126.

R.

Rabbit, embryology, 588.
RABL, fixing methods, 32, 50; embryos of *Salamandra*, 602; staining method, 256; cytological methods, 637, 639.
RABL-RÜCKHARD, embryology of Teleostea, 609.
RAMÓN Y CAJAL, *see* CAJAL.
Rana, embryology, 603.
RANVIER, alcohol, absolute, preparation of, 63; one-third ditto, 63; areolar tissue, 786; bladder of frog, 677; bone, 796; carmine,

The numbers refer to the Paragraphs, not to the Pages.

neutral, 164; cartilage, 45, 801; cornea, 658; corpuscles of Golgi, 671; decalcification, 563; epithelium, 646; gland-cells, 809—811; gold chloride impregnation, 225, 226 (and *see* Motor plates, Nerve-fibres, &c.); hæmatoxylin, 187; *injection masses*, carmine, 466; Prussian blue, 475, 476, 507; silver nitrate, 496; glycerin, 505; aqueous, 507; iodised serum, 360; maceration, 360, 523—534; medullated nerve, 720; motor plates, 667—668; mucus cells, 809; nerve-fibres, intra-epidermic, 649; neutral carmine, 164; nitrate of silver impregnation, 212, 213, 668; perruthenic acid, 809; picro-carmine, 159, 160; purpurin, 201; quinoleïn, 126; retina, 682, 685, tactile hairs, 647.

Ranvier and Vignal, osmium mixture, 29.

Rauvarienne, la, 107.

Reagents, how to procure, 94; selected collections of, 863.

Recklinghausen, von, impregnation methods, 210, 213, 214.

Reconstruction of objects from sections, 587.

Redding, gold process, 229.

Redwood nerve stain, 694.

Rehm, nerve stains, 725, 751, 752, 753.

Reich, silver nitrate, 213.

Reichenbach, embryology of *Astacus*, 625.

Reinke, staining horny tissues, 646.

Remak's copper hardening fluid, 82.

Renaut, hæmatoxylin, 189; hæmatoxylic eosin, 252; cornea, 658.

Renson, spermatological methods, 645.

Reptilia, embryology, 595 *et seq.*; myelon, 742, 784.

Resegotti, coal tar stains, 98, 101, 107, 258; staining by Substitution, 98; on the kinds of safranin, and on safranin staining, 101.

Resin, mounting media, 417, 422, 423; imbedding masses, 311—313; cement, 444, 445.

Retina, 21, 682 *et seq.*; fixing, 53, 78, 682; staining, 117, 683, 715; sections, 684; dissociation, 685.

Retterer, smooth muscle, 681.

Retzius, methylen blue method, 117.

Rezzonico, medullated nerve, 704, 720.

Rhabdocœla, 834.

Rhizophysa, 854.

Rhopalea, 811.

Rhumbler, methyl green and eosin vital stain, 261a.

Ribbon section-cutting, 281.

Ribesin, 209.

Richard, Bryozoa, 15.

Rindfleisch, maceration, 541.

Ringing wet mounts, 431, 432.

Ripart and Petit, preservative fluid, 64, 386.

Ripening hæmatoxylin solutions, 174—176.

Ritter, embryology of *Chironomus*, 620.

Robert, fixing *Aplysia*, 813, note.

Robertson, imbedding mass, 308.

Robin, injection-masses, 458—465, 521; and *see* Injection-masses.

Robinski, silver nitrate, 213.

Roboz, Zoltán von, alum-carmine, 155.

Rocellin, 95, 107.

Roche alum, 190, note.

Rocking microtome, 271, 867.

Rollett, carminroth, 167; freezing process, 320; celloidin imbedding, 301; cornea, 533, 658, 659; sections of muscle, 661.

Roosevelt, pyrogallate of iron, 232.

Rose B. à l'eau, 135.

Rose de naphthaline, 95, 107.

Röse, preparation of bone, 796.

Rosein, 95, 107.

Rosenthal, hydrochlorate of chinolin, 377.

Rossi, stain for nervous centres, 698; blood, 803.

Rössler, Phalangida, 828.

Rotatoria, 93, 839.

Rouge fluorescent, 95, 107.

Rouget, silver nitrate, 213, 214.

The numbers refer to the Paragraphs, not to the Pages.

ROUSSELET, permanent preservation of Rotifers, 839.
Rubidin, 95, 107.
Rubin, 95, 98, 107, 259.
RUFFINI, corpuscles of Golgi, 673.
RUGE, imbedding mass, 314.
RUNGE, imbedding mass, 314.
RUTHERFORD, picro-carmine, 162; hardening fluid, 735.
RYDER, double imbedding, 306; sexual gland of Lamellibranchiata, 645.

S.

SACHS, motor plates, 670; hæmatoxylin nerve stain, 696.
SAEFFTIGEN, *Echinorhyncus*, 837.
Saffron for staining, 204.
Safranin, 95, 98, 100, 101, 775; HENNEGUY'S method, 106; BABES', 638; FLEMMING'S new method, 258; for elastic tissue, 795; for cartilage, 801.
Sargartia, 849.
Sagitta, 217.
SAHLI, balsam, 418; nerve-centres, 730, 731; stain for, 773.
SALA, Golgi's silver chromate impregnation, 718.
Salamandra, embryology, 602; cytology of, 633, 637.
Salicylic vinegar, 391, 392.
Saliva, artificial, 531.
Salivary glands, 252, 528.
Salpa, 45, 811.
Salt solution, 359; for maceration, 526, 527.
SAMASSA, Golgi's silver chromate method, 709; sections of Ctenophora, 855.
Sandal-wood oil, 349.
SANFELICE, hæmatoxylin, 191.
SANKEY, staining nervous centres, 757.
Sapphirina, 822.
SARASIN, embryology of Reptilia, 597.
Sarcolemma, 664, 827.
SARGENT, injection method, 500; bleaching, 577.
SATTLER, silver staining, 214.
Säurefuchsin, 95, 98, 131; for nerves, 722, 772, 773.
Säuregelb, 95, 139.
SAVILLE KENT, *see* KENT.
SAWTSCHENKO, carcinoma corpuscles, 101.
SAZEPIN, antennæ, 824.
SCHÄFER, muscle-cells, 663; Weigert's nerve stain, 693.
SCHAFFER, cartilage, 801; retina, 683; reconstructing sections, 587.
SCHÄLLIBAUM, serial sections, 325.
SCHENK, fixing fluid, 65; carbolic fuchsin, 107.
SCHERING'S celloidin, 297.
SCHEWIAKOFF, Protozoa, 859.
SCHIEFFERDECKER, cartilage, 801; celloidin imbedding, 297—301; double stains, 265—267; injection masses, 513; levulose for mounting, 402; methyl mixture, 548, 685; pancreatin digestion fluid, 553; serial sections, 324, 337; retina, 548, 682, 685; skin and lymphatics, 688; gold staining nervous centres, 767; palladium ditto, *ibid.*; maceration of ditto, 781; medullated nerve, 720, 724; Mastzellen, 791; mucus cells, 808; muscle cells, 663; the water section-fixing process, 324.
SCHMAUS, uranium carmine, 167; staining nervous tissue, 747, 757.
SCHMIDT, embryology of *Limax*, 615; myelon of reptiles, 784.
SCHNEIDER, aceto-carmine, 158.
SCHOLTZ, *intra vitam* staining, 93.
SCHOTTLÄNDER, cytological methods, 643.
SCHULGIN, double stain, 243; embryology of *Amarœcium*, 612; paraffin mass, 287.
SCHULTZE, F. E., palladium chloride, 841, 234; embryology of Spongiæ, 856; Pennatulidæ, 849; section-stretcher, 280; hardening skin, 646.
SCHULTZE, MAX, acetate of potash mounting medium, 374; sulphuric

The numbers refer to the Paragraphs, not to the Pages.

acid macerating mixture, 546; retina, 685; iodised serum, 360.
SCHÜRMAYER, Infusoria, 858.
SCHÜTZ, Zacharias' carmine stain, 875.
SCHWALBE, smooth muscle, 676; cochlea, 686; impregnation, 210.
SCHWANN, sheath of, 719.
SCHWARZE, *Cercaria*, 833.
SCHWEIGGER-SEIDEL, carmine, 167.
Scorpions, eyes of, 579.
SCOTT and OSBORN, embryology of *Triton*, 600.
Sealing-wax varnish, 449.
SEAMAN, cement, 448; glycerin jelly, 411.
Sections, chain or ribbon, 281; collodionisation, 282; serial mounting, 323 *et seq.*; section-stretching and section-stretchers, 280; unrolling, 280; shrinkage in, 280; reconstruction, 587.
SEHRWALD, Golgi's silver chromate method, 708, 709, 711.
SEILER, cleaning slides, 864; mounting method, 355, 419; double stain, 241.
SELENKA, imbedding method, 273, 279, 314.
Sepia, eyes, 816.
Serial section mounting, 323 *et seq.*, 777, 778.
Serum, artificial, 361, 362, 524; iodised, 360, 361, 523, 524.
Shell, 311—313, 817, 819.
Shellac, cement, 448; imbedding mass, 310; fixative for sections, 327, 777; varnish, 448.
SHIMER, mounting medium, 398.
Shrinkage in elements of sections, 280.
Siebdosen, 863.
Sieve-dishes, 863.
Silver chromate impregnation, 704 *et seq.*, and *see* GOLGI.
Silver nitrate, *see* Nitrate of silver.
Siphonophora, 11, 854.
Sipunculus, 838.
Skin, hardening, 646; nerves of, 648.
Slides, cleaning, 864; labelling, 865.
SMITH, HOPEWELL, decalcification and sections of teeth, 565, 796, 797.
Soap, imbedding masses, 288 *et seq.*; 833.
Soda, solution for maceration, 529.
Soda, hypochlorite, *see* Eau de Labarraque.
Soda-carmine, 167.
Solferino, 95, 107.
SOLGER, sarcolemma, 664.
Solid green, 95, 105, 107.
SOLLAS, gelatin imbedding (freezing method), 319.
Solution of ERLICKI, of MÜLLER, of MERKEL, &c., see the names of the respective authors; normal salt, 359; macerating salt, 526, 527.
Solutions of metallic salts, how to keep, 25, 211.
SOMMER, *Macrotoma*, 829.
SOUZA, DE, pyridin, 90.
SPEE, prepared paraffin, 286.
SPEK, VAN DER, and UNNA, plasma-cells, 792.
Spermatological methods, 645.
Spermatozoa, preparation, 645.
Sphyranura, 833.
Spicules of sponges, 856.
Spinal cord, 728, 743, 767, 880 *et seq.*
Spirographis, 841.
Spongiæ, 856.
Spongilla, 856.
SQUIRE, histological reagents, 94; oil of origanum, or white thyme, 348; methyl green, 109; commercial salts of gold, 221; glycerin jelly, 413; damar medium, 420; granule cells, 788; mounting methyl-green stains, 879; decalcification, 566, 572; Congo red for nerves, 724; picro-carmine, 162; hæmatoxylin, 188a.
STAHL, immobilisation of Infusoria, 858.
Staining, generalities, 91—94; choice of stain, 94; on slide, 2; *in toto*, 4, 5; *intra vitam*, 93, 114, 115, 634, 858; reagents, how to obtain, 94; staining by substitution, 98; directions for the Indirect Method, 96—100; staining nerve-tissue,

The numbers refer to the Paragraphs, not to the Pages.

see Nerves, Central Nervous System, Neurological Methods, &c.
Stains, General, Selective (Nuclear, Plasmatic, and Histologically Specific), defined, 91; aqueous and alcoholic stains, 5; effect of transparency and opacity of stains, 100; Generalities, 91 *et seq.*, and *see* Anilin black, Anilin blue, Carmine, Cochineal, Eosin, Gold Hæmatoxylin, Indulin, Iron, Orchella, Purpurin, Saffron, Safranin Silver, &c.
Stains, combination, in general, 235 *et seq.*; *anilin blue* and carmine, 243; *borax-carmine* and indigo, 241, 242; ditto and picro-carmine, 238; *carmine* and anilin blue or bleu de Lyon, 243; ditto and malachite green, 244; ditto and methyl green, 245; *carmine* and picric acid, 236—240; *carmine* and metallic stains, 155, 249; *dahlia* and Säurefuchsin, 258; *Ehrlich-Biondi-Heidenhain* mixture, 259, 639; *eosin* and anilin green, 265; ditto and dahlia, 258, 266; ditto and hæmatoxylin, 251, 252; ditto and iodine green, 270; ditto and methyl green, 260—261a; ditto and methyl violet, 267; ditto and gentian violet, 258; *fuchsin* and methylen blue, 268; *gentian violet* and eosin, 258; ditto and safranin and orange, *ibid.*; *gold chloride* and other stains, 229; *hæmatoxylin* and delta- or benzo-purpurin, 131, 251, 253; ditto and eosin, 251, 252; ditto and iodine green, 255; ditto and nitrate of rosanilin, 255; ditto and picric acid, 236; ditto and rubin and orange, 254; ditto and safranin, 256; *iodine green* and Bengal rose, 270; ditto and hæmatoxylin, 255; *lithium-carmine* and picric acid, 240; *methyl-green* combinations, 245, 259—263; *methyl-violet* and eosin, 258, 267; *picro-carmine*, 159 *et seq.*, 237; ditto and iodine green, 246, 247; ditto and methyl green, 246; ditto and borax-carmine, 238; ditto and other anilins, 247; *safranin* and anilin blue, 270; ditto and gentian and orange, 258; ditto and hæmatoxylin, 256; ditto and gold chloride, 270; ditto and indigo, 249; ditto and methylen blue, 774; ditto and nigrosin, 269; ditto and Säurefuchsin and methylen blue, 773.
Starch injection mass, 518.
Stentor, 858.
STEPHENSON, mounting medium, 414.
Sternaspidæ, 841.
STIEDA, cements, 451; clearing media, 343.
STILLING, macerating brain, 781.
STILLING and PFITZNER, stomach of *Triton*, 680.
STIRLING, double stains, 246, 255; sulphocyanides of ammonium and potassium, 530; anilin blue-black, 757.
STOHR, double stain, 251.
Stomach of *Triton*, 643, 680.
Storax, 427.
Stoves and water-baths, 277.
STRAHL, embryology of Reptilia, 595.
STRASBURGER, cytological methods, 643.
STRASSER, plastic reconstruction, 587; section-stretcher, 280; serial sections, 325, 326, 340.
STRICKER, imbedding mass, 309.
Strychnin for killing, 18, 858.
Styrax, 427.
Sublimate, corrosive, *see* Corrosive sublimate.
Substitution of stains, 98, 102, 107.
SUCHANNEK, the water section-fixing process, 324; bergamot oil, 347; anilin oil for clearing, 353; Venice turpentine, 423; Sieve-dishes, 863.
Sulphate of copper fluids, 44, 82; impregnation, 234.
Sulphate of iron for fixing, 834.
Sulphindigotate of soda or potash, 202.
Sulphocyanides, 530.
Sulphuric acid for maceration, 546.

The numbers refer to the Paragraphs, not to the Pages.

Sulphurous acid, for Infusoria, 860; for bleaching, 874.
SUMMERS, serial sections, 325, 337.
"Sunning" solutions of metallic salts, 25, 211.
SUSSDORF, mucus cells, 807.
SWAEN and MASQUELIN, spermatological methods, 645.
Sycandra, 856.
Sympodium, 849.
Synapta, 845.
Syncoryne, 851.
Syrup, imbedding masses, 308, 316, 317; examination media, 365.

T.

Tactile corpuscles, 651 *et seq.*
Tactile hairs, 647.
Tænia, 832; embryology of, 628.
TAENZER, elastic tissue, 795.
TAFANI, inner ear, 687.
TAGUCHI, injection, 512.
TAIT, LAWSON, litmus stain, 209.
TAL, Golgi's mercury stain, 760.
Tannin solution, 387; for Infusoria, 860.
TARTUFERI, impregnation, corneal cells, 659.
Tegumentary organs of Vertebrates, 646 *et seq.*
TRICHMANN, white injection, 492; oil mass, 519.
Teleostea, embryology of, 604 *et seq.*
Tendon, 671 *et seq.*
Tethya, 856.
THANHOFFER, VON, silver staining, 214; sarcolemma, 827; brain-tissue, 782.
Thenea, 217.
Thermostats, 277.
THIERSCH, carmine injection, 469; Prussian blue ditto, 479; lead chromate ditto, 483; green ditto, 488; oxalic acid indigo-carmine, 203.
Thimbles, paper, to make, 273.
THIN, retina, 685.
Thionin, for plasma-cells, 794; for mucin, 807.
Thiophen green, 141.
THOMA, microtome, 271; celloidin imbedding, 301; decalcification, 561; egg imbedding mass, 314.
THOMPSON'S high refractive medium, 416.
THRELFALL, section-fixing method, 336.
THWAITES, preservative fluid, 369.
Thyme, oil of, 305.
Tiara, 851.
Tintinnodea, 859.
TIZZONI, alum-carmine, 153; cytological methods, 643; medullated nerves, 720; hardening skin, 646.
Tobacco-smoke killing method, 9.
TOISON, blood, 804.
Tolu balsam, mounting medium, 428; cement, 450.
Toluidin blue, 123.
Toluol, 275, 283, 354; for preserving, 2.
Tongue of frog, 657.
TORNIER, hæmatoxylin staining, 193.
TOURNEUX and HERMANN, silver staining, 213; epithelium, 646.
Trays, paper, to make, 273.
Trematodes, 833.
TRENKMANN, stains for flagella, 861.
Triænophorus, 832.
Trichinæ, 881.
TRINCHESE, nerve-endings in muscle, 667.
Triton, eye, 682; stomach, 643, 680; ova, 600, 601.
Tropæolin, 95, 107, 139.
Trypsin digestion fluid, 554.
TEZEBINSKI, spinal cord, 732.
TSCHISCH, nerve-centres, 77.
TUBBY, celloidin imbedding, 298.
TULLBERG, narcotisation with magnesium chloride, 17.
Tunicata, 811; embryology of, 611, 612.
Turbellaria, 834.
Turpentine, for clearing, 350; for mounting, 423; cement, 444.

U.

ULIANIN, embryology of Amphipoda, 626.

The numbers refer to the Paragraphs, not to the Pages.

UNDERWOOD, gold process, 228.
Unio, 118.
UNNA, bleaching chromic objects, 30; ripening of hæmatoxylin solutions, 174, 175, 176; instanteously ripe hæmatoxylin stain, 175; half-ripe stock solution, 176; plasma-cells and Mastzellen, 792; elastic tissue, 795.
UPSON, gold and iron method, 770; gold and vanadium method, 771; other stains, 748.
Uranium, acetate, 28, 65.
Uranium, nitrate, 78.
Uranium - carmine, GIERKE and SCHMAUS, 167.
Urodela, embryology of, 598—602.
USKOFF, nitric acid in cytology, 643.
USSOW, embryology of Cephalopoda, 613.

V.

Vacuum imbedding, 278.
VALETTE, ST. GEORGE v. LA, spermatological methods, 634.
VAN BENEDEN, *see* BENEDEN, VAN.
VAN GIESON, clearing with anilin oil, 305.
VAN HEURCK, mounting medium, 415.
Vanadate of ammonium, 772; injection mass, 511.
Vanadium, perchloride, 683, 726.
Varnish, 430 *et seq.*; amber, 446, 447; asphalt, 436; Brunswick black, 437; copal, 424, 447; negative, 426; sealing-wax, 449; shellac, 448.
VASALE, hæmatoxylin nerve stain, 695.
Vegetable wax, 287.
VEJAS, staining nervous centres, 757.
Velella, 854.
Venice turpentine, for mounting, 423; cement, 444.
Veretillum, 8.
Veridin, 109.
Vermes, 832—844; embryology, 628 *et seq.*
Vert d'alcali, 109.
Vert d'Eusèbe, 109.
Vert en cristaux, 109.
Vert lumière, 109.
VERWORN, narcotisation, 14.
Vesuvin, 95, 98, 110.
VIALLANES, fixation of Insecta, 28; gold method, 227; celloidin imbedding, 301; brain of Arthropods, 825; osmic acid mixture, 28.
VIALLETON, embryology of Aves, 594.
Victoria blue, 95, 98, 104, 795.
VIGNAL and RANVIER, osmium mixture, 28; picro-carmine, 160.
VILLE, carmine injection, 467.
Violet B., 95, 129, 786.
VIRCHOW, action of light on chromic objects, 30.
VIVANTE, bone, 799.
VOGT and YUNG, *Cucumaria*, 845; Rotifers, 839; *Sipunculus*, 838; cleansing intestine of Lumbricus, 840.
VON EBNER, decalcification mixture, 563.
VON LENHOSSÉK, nerves of Annelids, 843; myelon of reptiles, 784.
VON MÄHRENTHAL, osmium impregnation, 233.
VOSMAER, epithelium of sponges, 217.
VOSSELER, Mayer's albumen fixative, 328; Venice turpentine, 423; wax feet, 522; cardboard ridges for slides, 865.

W.

WADDINGTON, arabin, 329; Infusoria, 860.
Wagnerella, 574.
WALDEYER, inner ear, 687; plasma-cells, 788; decalcification, 559.
Walnut-juice stain, 209.
WARD, narcotisation methods, 19; *Sipunculus*, 838.
Washing out, 2, 22, 24.
Washing osmic acid objects, 29.
WATASE, embryology of Cephalopoda, 613.
Water, fresh or warm, for killing, 20.

The numbers refer to the Paragraphs, not to the Pages.

Water-baths, 277.
WATNEY, dichroism of hæmatoxylin stains, 178.
Wax imbedding masses, 287.
Wax feet, 522.
WEBB, dextrin freezing mass, 318.
WEBSTER, naphtha for paraffin imbedding, 275.
WEBER, Rotifers, 839.
WEDL, orchella stain, 206.
WEIGERT, Bismarck brown, 110; picro-carmine, 161; hæmatoxylin nerve methods, 688—703, 755 (the 1885 method, 688; the 1891 method, 703; variations, 689—702, 755); ditto for retina, 683; clearing celloidin sections, 305, 703; mounting serial sections, 305, 340, 426, 703; varnish for mounting without cover, 426; hardening nerve-centres, 730; Säurefuchsin for ditto, 772.
WEIL, section method, 313; bone, 796.
WHEELER, embryology of Blattida, 619.
WHITE, sections of bone or dental tissue, 796.
White lead cement, 453.
White of egg, section-fixing process, 328; imbedding methods, 314, 321; injection-mass, 498.
White zinc cement, 451.
WHITMAN, fixing mixture, 83; ova of Amphibia, 598; pelagic ova, 610; Hirudinea, 844.
WICKERSHEIMER, preservative fluid, 390.
WILL, Aphides, 621.
WILSON, Alcyonaria, 849; embryology of Lumbricus, 630.
WISSOWSKY, blood, 804.
WITT, shellac, 448.
WOLFF, motor plates, 670; bladder of frog, 677.
WOLTERS, hæmatoxylin nerve stains, 699, 700, 726; cartilage, 801; skin nerves, 648; elastic tissue, 795.
WOODWARD, borax-carmine, 167.
WRIGHT, *Sphyranura*, 833.

X.

Xylol, for clearing, paraffin sections, 354; ditto celloidin sections, 354; for half-clearing brain sections, 779; for paraffin imbedding, 275; for preserving tissues, 2.

Z.

ZACHARIAS, acetic alcohol, 54; acetic carmine, 158, 875; ovum of *Ascaris*, 644.
ZENTHOEFER, elastic tissue, 795.
ZIEGLER, white cement, 452.
ZIEHEN, gold and mercury impregnation method, 769.
ZIEHL'S carbolic fuchsin, 107.
ZIMMERMANN, microtomes, 271.
ZIMMERMANN Sieve-dishes, 863.
Zinc chloride, for hardening brain, 780; impregnation method, 762.
Zinc white cement, 451.
Zoantharia, 849.
ZOJA, methylen blue, 114; bioblasts of Altmann, 641; *Hydra*, 850.
ZOLTÀN VON ROBOZ, alum-carmine, 155.
Zoobothrium, 812.
ZSCHOKKE, deltapurpurin, benzopurpurin, 133, 253.
ZUPPINGER, ganglion cells, 756.
ZWAARDEMAKER, safranin stain, 101; cytological method, 643.

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